

# **Conservation and potential for recovery of freshwater mussels in Mediterranean-climate regions**

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BSc (Hons)



This thesis is presented for the degree of Doctor of Philosophy of  
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Centre for Natural Resource Management (Albany Campus)  
and School of Agriculture and Environment

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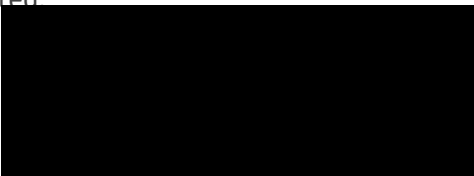
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## Abstract

Despite the ongoing biodiversity crisis in freshwater ecosystems, opportunities exist for conservation and restoration through effective management. Freshwater mussels are highly vulnerable to disturbances associated with anthropogenic development. Mussel populations are also unlikely to recover quickly from decline, as they are long-lived (sometimes >100 years), slow growing, and late to mature. A growing body of work is showing that mussels are an important functional component of freshwater ecosystems, providing numerous goods and services to human populations, so there is growing concern over the effect that their global decline will have on humanity.

In places that have been extensively altered by human settlements, such as Mediterranean-climate (med-) regions, freshwater mussels are likely to be particularly threatened, and have less potential to recover than in other places. The med-region of southwestern Australia has just one species of freshwater mussel, the endemic *Westralunio carteri*. Although widespread and often locally abundant, many *W. carteri* populations have declined or been lost, and the species is listed as vulnerable to extinction due to a recent dramatic contraction in range.

The aims of this thesis were to (1) determine if the plight of *W. carteri* is typical of mussels in med-regions, and (2) to use *W. carteri* as a case study to assess the potential for med-mussels to recover following decline. To achieve these aims, four strongly linked research components were undertaken, incorporating an extensive global desktop study, in combination with field surveys of various mussel populations to determine contemporary (e.g. presence/absence, abundance) and historical (i.e. phylogeographic) demographic patterns at both local and landscape scales.

This thesis shows that low mussel species-richness is a common trait for freshwater ecosystems in med-regions, and that med-mussels are more imperiled than other mussel species globally. Encouragingly, in southwestern Australia, there is evidence of *W. carteri* population recovery in response to conservation actions at various scales. These include re-expansion into upstream habitat following the installation of a fishway, and the recolonisation of a salinised reach in response to improved catchment management. Moreover, in these instances recovery appears to have begun soon after mitigation of the original threat. Mussels in the region have also persisted through historical climate change via contraction to refugia in multiple locations.

Mussel decline is likely to have significant impacts on ecosystem services provided by this important functional group. That being the case, low mussel species richness in med-regions increases the

importance of each species, as there is little or no redundancy within the group if a species is lost. These findings demonstrate that there is an urgent need to increase conservation effort aimed at mussels in med-regions. Remnant 'pristine' systems hold great value, as they are likely to have important environmental resources that buffer mussel populations against future disturbances. However, as the majority of med-regions are highly altered, most conservation opportunities will involve mitigating threats in degraded habitats. The fact that mussel populations can begin to recover quite rapidly increases the incentive to commence restoration actions on declining populations; however, the time to 'full' recovery is unknown and this has yet to be observed. This highlights that delaying the implementation of restoration actions will only prolong what is likely to be a long recovery process, and that prevention is better than cure in the case of mussel conservation.

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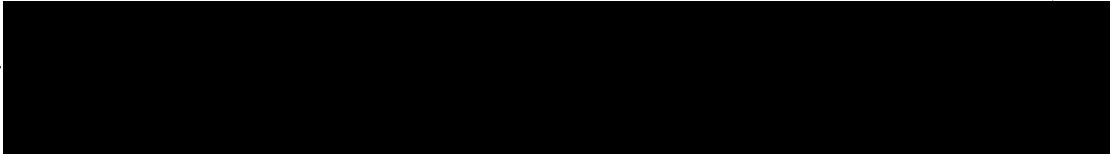
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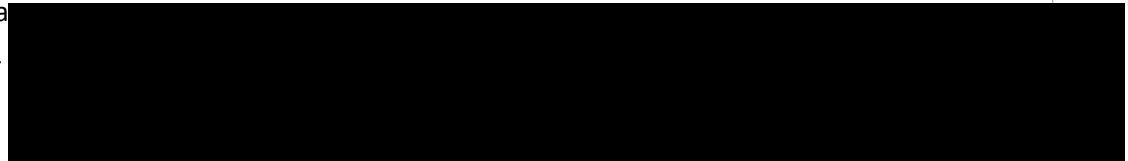


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## Authorship declaration: Co-authored publications

This thesis contains work that has been published and work that has been submitted for publication.

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| Details of the work:<br>Benson, J.A., Stewart, B.A., Close, P.G. & Lymbery, A.J. (2021). Freshwater mussels in Mediterranean-climate regions: Species richness, conservation status, threats, and conservation actions needed. <i>Aquatic Conservation: Marine and Freshwater Ecosystems</i> , 31(3), 708-728.<br><a href="https://doi.org/10.1002/aqc.3511">https://doi.org/10.1002/aqc.3511</a> |
| Location in thesis:<br>Chapter 2  |
| Student contribution to work:<br>Justin Aaron Benson conceived the idea (100%), collected the data (100%), performed the analysis (100%), and wrote the manuscript (90%) with edits and direction provided by supervisors.  |
| Co-author sign <br>[insert signature<br>28.7.2021  |

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Chapter 1:  
General introduction



*Cover picture: Freshwater mussels and various rivers in southwestern Australia*

## **Freshwater biodiversity conservation in Mediterranean-climate regions**

Freshwater ecosystems support approximately 10% of all described species despite covering only a small fraction (<1%) of the Earth's surface (Strayer & Dudgeon, 2010). These ecosystems are especially vulnerable to environmental change while being focal points of anthropogenic development (Dudgeon et al., 2006). The growing reliance of human populations on freshwater resources places increasing pressure on freshwater biodiversity, which is being lost at a rate far greater than that in terrestrial and marine ecosystems (Sala et al., 2000; Vörösmarty et al., 2010). As the impacts of species loss on ecosystem resilience are expected to be non-linear (Scheffer et al., 2001; Folke et al., 2004), there is an urgent need to address the current decline by exploring opportunities for conservation and restoration through effective mitigation of threats (Dudgeon et al., 2006; Reid et al., 2019; Tickner et al., 2020). Although the primary threats driving freshwater biodiversity loss are well known (e.g. invasive species, overexploitation, habitat degradation, pollution, and modified flows) (Dudgeon et al., 2006), and recently emerging threats have been identified (e.g. climate change, microplastics, and salinisation) (Reid et al., 2019), the capacity for freshwater taxa to recover following the mitigation of threats is often not assessed (Tickner et al., 2020). In many cases, conservation actions directed at terrestrial systems are simply expected to have inevitable trickle down benefits for freshwater ecosystems (Tickner et al., 2020). Where the benefits to freshwater taxa are assessed, the focus is often on large, charismatic 'flagship' species such as large fish, reptiles, and mammals (Carrizo et al., 2017). This potentially neglects taxa that fall outside of the public consciousness that may nonetheless be important ecological components of these ecosystems (Geist & Hawkins, 2016). Further, these unrecognised species might serve as ideal flagships if public awareness of their importance could be increased (Verissimo, MacMillan & Smith, 2011).

Freshwater biodiversity loss is likely to occur more rapidly in regions where more people live (Vörösmarty et al., 2010). Mediterranean-climate (med-) regions (the Mediterranean basin, coastal California, central Chile, the Cape Floristic Region of South Africa, and southern Australia) are perhaps the best example of this, as they are magnets for human settlement, resulting in extensive landscape modification and exploitation of freshwater ecosystems (Aschmann, 1973). Freshwater biodiversity in med-regions has been subjected to a range of threats at various scales, including local (e.g. in-stream barriers and point-source pollution), landscape (e.g. agriculture and urbanisation), and global (invasive species and ongoing climate-change) (Bonada & Resh, 2013; Cooper et al., 2013; Figueroa et al., 2013; Filipe, Lawrence & Bonada, 2013; Ellender et al., 2017). As a result, freshwater biodiversity in these

regions has declined at a rate that is unparalleled by other climate-type regions (Moyle & Leidy, 1992; Cooper et al., 2013). Populations of many freshwater taxa have retreated to areas of remnant freshwater refuge (Beatty et al., 2011), while others persist within suitable pockets of the degraded landscape, including 'novel' anthropogenic systems (Chester & Robson, 2013; Sousa et al., 2021). 'Pristine' remnant systems retain great conservation value, as they are likely to contain important environmental resources that will buffer populations against future disturbances; however, due to the rarity of such systems, degraded areas must also receive consideration for protection and management. Further, restoration actions that encourage re-expansion of restricted populations, whether from degraded or remnant refuges, will enhance resilience to future disturbances by increasing the available habitat and population sizes.

### **Freshwater mussels: A case study for med-region conservation and restoration**

Freshwater mussels (Bivalvia, Unionida; hereafter 'mussels') are experiencing a global decline that is expected to significantly alter the functioning of freshwater ecosystems (Lydeard et al., 2004; Vaughn, 2010). Mussels are important ecosystem engineers, and often congregate in dense multi-species beds that have significant impacts on various ecological processes (Vaughn & Hakenkamp, 2001; Howard & Cuffey, 2006; Aldridge, Fayle & Jackson, 2007; Spooner & Vaughn, 2008; Vaughn, 2010; Vaughn, Atkinson & Julian, 2015). For example, as filter feeders they can improve water clarity and quality by removing suspended sediments, nutrients, and algae (Vaughn & Hakenkamp, 2001; Atkinson et al., 2013) and subsequent biodeposition creates nutrient-rich hotspots that benefit benthic food webs and alter community structure (Vaughn & Spooner, 2006; Atkinson & Vaughn, 2015). While mussel burrowing behavior mixes and aerates the sediment (Collier et al., 2018), large mussel beds can also reduce shear stress at the sediment-water interface (Sansom et al., 2020), and provide physical habitat for other organisms (Howard & Cuffey, 2006; Aldridge, Fayle & Jackson, 2007). Although the causes of mussel decline and the best methods for reversing it are not always known (Strayer et al., 2004), many of the obvious threats (e.g. pollution, landscape modification, climate change (Lopes-Lima et al., 2018)) are the same as those driving the more general loss of freshwater biodiversity in med-regions. Mussel species have narrow environmental tolerances for habitat quality, but as they are largely immobile, they often have little capacity to avoid threats in their local environment (Gough, Landis & Stoeckel, 2012). They also have a complex life cycle, including a parasitic larval stage, which relies on a host-fish for development and dispersal (Modesto et al., 2018). This being the case, fish-species declines and barriers to fish movement can wipe out mussel populations (Watters, 1996; Brainwood, Burgin & Byrne, 2008).

As mussels are slow growing, long lived (sometimes >100 years), and late to mature, populations are unlikely to recover quickly (Strayer et al., 2004), and the potential for populations to recover at all is still mostly unstudied (although see Sietman et al., 2001; Riccardi et al., 2016; Gillis et al., 2017).

## ***Westralunio carteri*: The only freshwater mussel species in southwestern Australia**

*Westralunio carteri* is the only freshwater mussel species in the med-region of southwestern Australia (SWA) (Walker, Jones & Klunzinger, 2014). As with many other freshwater taxa in SWA, *W. carteri* has ancient origins and is endemic to the region (Davies & Stewart, 2013; Rix et al., 2015), and has also been highly impacted by threats such as landuse change, stream modifications, overexploitation of water resources, and ongoing aridification (Robson et al., 2013; Pettit et al., 2015). Although *W. carteri* remains widespread, and is sometimes locally abundant, it is listed as Vulnerable (VU A2c) on the International Union for Conservation of Nature Red List due to a dramatic reduction in range (~49%) over the last three generations (Klunzinger et al., 2015; Klunzinger & Walker, 2020). The decline of *W. carteri* may be particularly important for freshwater ecosystems in SWA because, as the only mussel species known to occur in the region, its loss would result in the loss of all mussel-derived ecosystem services.

In recent years, attempts have been made to restore SWA's waterways and the broader landscape through actions such as legislated control on land clearing, targeted revegetation of agricultural areas and riparian zones, and the reinstatement of environmental flows (Mayer, Ruprecht, & Bari, 2005; Pettit et al., 2015). These actions have mostly been driven by the need to halt economic losses associated with degradation of water resources, farming land, rural infrastructure (Mayer, Ruprecht, & Bari, 2005; Pettit et al., 2015). Nonetheless, these actions may provide freshwater taxa in the region with an opportunity to recover, but evidence of this having occurred is still required.

## **Knowledge gaps, thesis structure and research aims**

Although mussels are recognised as important functional components in freshwater ecosystems, and have experienced a dramatic global decline, there has been no synthesis of existing data on mussels that occur within med-region boundaries, and the potential for population recovery remains mostly unassessed. The aims of this thesis were to (1) determine if the plight of *W. carteri* is typical of mussels in med-regions (med-mussels), and (2) to use *W. carteri* as a case study to assess the potential for med-mussels to recover following decline. To achieve these aims, I undertook four interlinked research



components (Chapters 2-5), beginning with a comprehensive literature review of the mussel species that occur in med-regions and various aspects of their conservation (Chapter 2). This was followed by three separate field studies to determine if mussel populations showed evidence of having recovered from disturbances at various spatial and temporal scales (Chapters 3-5). Background and justification for these research components has been provided here in Chapter 1, while the key findings are integrated and briefly summarised in the context of freshwater conservation in med-regions in the general conclusion in Chapter 6. The context and specific aims of each research component are outlined below.

## ***Chapter 2***

Despite the alarming decline of mussels globally (Lydeard et al., 2004; Lopes-Lima et al., 2018; Ferreira-Rodriguez et al., 2019), and the increasing threat to freshwater biodiversity in med-regions specifically (Bonada & Resh, 2013; Filipe, Lawrence & Bonada, 2013; Robson et al., 2013), there has been no synthesis of the plight of mussels within all med-regions. In this chapter, I review data from the International Union for Conservation of Nature (IUCN) Red List (<https://www.iucnredlist.org/>) in order to compare the conservation status, threats, and conservation actions needed for med-mussels with that of other freshwater mussel species. I also present the first comprehensive catalogue of mussel species that occur in med-regions based on existing taxonomic and phylogeographic literature. The aim of this study was to assess med-mussel conservation relative to mussel species in other regions, and to test the following specific hypotheses:

- Med-mussels are more imperiled than other mussel species globally.
- Med-mussels face a wider range of threats than other mussel species globally.
- Med-mussels require greater conservation action than other mussel species globally.

## ***Chapter 3***

Instream barriers such as dams and weirs are a major driver of freshwater biodiversity loss as they can interrupt important migratory pathways and isolate taxa from essential habitats for reproduction and refuge (Rolls et al., 2013). Barrier removal is often desirable for conservation purposes; however, it is not always possible where the function of the barrier is still required (Gangloff, 2013). The construction of 'fishways' (or 'fish ladders') to bypass these barriers can restore connectivity for fish in some cases, but many fishways do not provide adequate passage for all freshwater fauna (Harris et al., 2016). As the mobility of mussels is mostly limited to dispersal on a host-fish during their larval stage, barriers to fish

movement can lead to the localised extinction of mussel populations (Watters, 1996; Brainwood, Burgin & Byrne, 2008). While fishways might be expected restore connectivity for mussel populations, and allow recolonisation of extirpated reaches, examples of this having occurred are a lacking. This being the case, the efficacy of fishways as a restoration strategy for mussels requires validation in the field. The aims of this study were:

- To determine whether the installation of a fishway had facilitated recolonisation by mussels upstream of a weir.
- To test the hypothesis that larger size classes would be absent from the upstream reach, reflecting a historical lack of recruitment caused by the weir.

#### ***Chapter 4***

Secondary salinisation is a major threat to freshwater biodiversity globally, and is particularly prevalent in med-regions (Degens et al., 2012; Robson et al., 2013; Estévez et al., 2019). Reversing the effects of secondary salinisation is a growing global challenge; however, there are few examples of species recovery following salinity reversal (Bathe & Coring, 2011; Arle & Wagner, 2013). In SWA, the landscape has been extensively cleared for agriculture, and as a result more than half of the large rivers in the region are now brackish or saline (Mayer, Ruprecht, & Bari, 2005; Beatty et al., 2011). Consequently, freshwater fauna in the region is often restricted to remnant freshwater tributaries closer to the coast (Beatty et al., 2011; Pettit et al., 2015). In recent years, secondary salinisation has been tackled through legislated control on land clearing, as well as revegetation of agricultural areas, and salinity has begun to reverse in some rivers (Mayer, Ruprecht, & Bari, 2005). This creates the potential for salt-sensitive fauna to recolonise formerly salinised areas, but there is no evidence for this having occurred (Robson et al., 2013). The objective of this study was to assess the potential for mussels to recover in a catchment that has been managed to reverse salinisation, and the following hypotheses were tested:

- Although mussels would be absent from high salinity sites, there would be evidence (i.e. old shells) of previous occupation at those sites.
- Long-term salinity data would show that salinity has begun to decline in the river.
- The decline in salinity has facilitated increased mussel recruitment and recolonisation in previously salinised habitats.
- Mussels would be more abundant in freshwater tributaries than in brackish sites, while being entirely absent in higher salinity areas.

## Chapter 5

Intraspecific genetic diversity provides species with the evolutionary potential to adapt to changing environments (Hoffmann & Sgro, 2011). ‘Hotspots’ of high intraspecific diversity and areas of historical refugia are key targets for the conservation of threatened taxa, as they often contain populations with greater evolutionary potential, and may provide important environmental resources that buffer those populations against disturbances (Canestrelli et al., 2014). The intraspecific genetic diversity of mesic taxa in SWA is not uniformly distributed, and many species comprise multiple divergent lineages with highly localised haplotypes due to contraction to separate refugia during past climate change (Byrne, 2008). There is some evidence that the south coast was buffered against aridity at those times by sustained higher rainfall, creating a climate refugia for biodiversity in that area (Hopper, 1979; Wardell-Johnson & Horwitz, 1996; Roberts et al., 1997; Hopper & Gioia, 2004; Nistelberger et al., 2014). Preliminary phylogeographic work on *W. carteri* has found evidence of genetic structuring among populations from the western, southwestern, and southern coasts (Klunzinger et al., 2021). Those authors hypothesised that these three genetic groups would have non-overlapping distributions, and proposed they be recognised as separate evolutionary significant units (ESUs) (Klunzinger et al., 2021). Given the clear implications for the conservation of mussels in SWA, greater sampling resolution is required to describe the boundaries between these lineages and the location of intraspecific genetic hotspots. The aim of this chapter was to assess the spatial distribution of *W. carteri* diversity at various scales using mitochondrial DNA. Samples were incorporated from every basin within the known range of this species. The following hypotheses were tested:

- *W. carteri* populations comprise three ESUs with non-overlapping distributions.
- The south coast ESU would have the highest genetic diversity of the three ESUs due to long-term stability on the South Coast.
- There would be a prevalence of private haplotypes throughout SWA due to recent lack of connectivity among river basins.

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## Chapter 2:

### Freshwater mussels in Mediterranean-climate regions: Species richness, conservation status, threats, and conservation actions needed



**Cover picture:** *The Kent River above Nile Creek. This ‘pristine’ reach is actually salinised, and no live freshwater mussels were found; only old shell fragments*

Benson, J.A., Stewart, B.A., Close, P.G. & Lymbery, A.J. (2021). Freshwater mussels in Mediterranean-climate regions: Species richness, conservation status, threats, and Conservation Actions Needed. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 31(3), 708-728.

<https://doi.org/10.1002/aqc.3511>

## **Abstract**

The five global Mediterranean-climate regions are experiencing alarming rates of freshwater biodiversity loss. Although freshwater mussels are recognised as important functional components in aquatic ecosystems, and are among the most threatened faunal groups globally, there has been no synthesis of the plight of this group within these regions. Data from the International Union for Conservation of Nature (IUCN) Red List were reviewed to compare the conservation status, threats, and conservation actions needed for freshwater mussel species occurring in Mediterranean-climate regions (med-mussels) with those of other freshwater mussel species globally. The first comprehensive catalogue of med-mussel species was compiled using existing taxonomic literature. There are 41 med-mussel species, 30 of which occur in the Mediterranean basin. Many regions have just a single species and regions where multiple species occur generally only have between one and four species per river basin. Med-mussel species are almost twice as likely to be 'Imperiled', are affected by 2.4 times more threats, and require 3.5 times more conservation actions than non-med mussels. In many cases, the exact threats have not been identified. In combination with low species richness, this level of imperilment means that Mediterranean-climate regions are at risk of losing the benefits that mussels provide to broader ecosystem functioning. The conservation of med-mussels can be improved by increasing knowledge of species distributions, including the identification of cryptic species and significant management units, through population genetic work. In addition, recognising the potential of 'novel' habitats and refuge areas could augment the management of this important functional group.

## Introduction

Freshwater ecosystems contribute significantly to global biodiversity, supporting approximately 10% of all described species despite covering <1% of the Earth's surface (Strayer & Dudgeon, 2010). Freshwater biodiversity plays an essential part in maintaining ecosystem function and also provides numerous goods and services for an expanding human population (i.e. ecosystem services) (Cardinale et al., 2012; Collen et al., 2014; Vaughn, 2018). As human reliance on freshwater resources grows so does the pressure on freshwater biodiversity, which is now declining at a rate that far exceeds that observed in terrestrial systems (Sala et al., 2000; Vörösmarty et al., 2010; Reid et al., 2019). Losses are likely to be both faster and more severely felt in regions where more people live (Vörösmarty et al., 2010), so effective conservation and management of these areas should be prioritised.

Mediterranean-climate (med-) regions (i.e., the Mediterranean basin, coastal California, central Chile, the Cape Floristic Region of South Africa, and southern Australia) are recognised hot spots for freshwater biodiversity (Aschmann, 1973; Bonada & Resh, 2013). Located at similar latitudes on the coastal fringes of continental land masses, the characteristic cycle of hot–dry summers and cool–wet winters in these regions creates a seasonal river hydrology that includes periods of flow and periods of low or no flow (Aschmann, 1973). The hydrological similarity of med-rivers has driven the evolution of comparable ecosystems in geographically separated locations (Bonada & Resh, 2013), and many of the freshwater species are endemic, often stemming from ancient lineages (Davies & Stewart, 2013; Figueroa et al., 2013). Freshwater research has not been evenly spread among the regions, with most of the focus being on the northern hemisphere, particularly in the European part of the Mediterranean basin (Ball et al., 2013; de Figueroa et al., 2013; de Moor & Day, 2013). Effective conservation of med-biodiversity will require robust knowledge of species distributions, taxonomic affinities, and the threats faced across all med-regions.

Med-regions are magnets for human settlement and have been greatly affected by numerous human disturbances (Bonada & Resh, 2013). Landscape modifications, resource exploitation, and other disturbances (e.g. pollution and non-native species) have caused freshwater biodiversity to decline at a rate that is unparalleled in other climate-type regions (Moyle & Leidy, 1992; Cooper et al., 2013). Recent increases in air temperature and lower rainfall have led to reduced annual run-off, placing greater demand on water resources (Cai & Cowan, 2008; Vicuña, Garreaud & McPhee, 2011; Filipe, Lawrence & Bonada, 2013), and climate projections suggest that all med-regions will become increasingly arid (Filipe, Lawrence & Bonada, 2013; Robson et al., 2013). The combination of existing and future threats will

place species in med-regions under increasing pressure, so effective conservation and management is essential.

Previous reviews of freshwater biodiversity conservation in med-regions have been limited to taxa within a single region. For example, researchers in southwestern Australia (Davies & Stewart, 2013) and California (Ball et al., 2013) suggested that the number of species assessed by the International Union for Conservation of Nature (IUCN) Red List of Threatened Species was not consistent with the extent of the threats in those regions. Similarly, reviews from Chile (Figueroa et al., 2013) and South Africa (de Moor & Day, 2013) discussed difficulties associated with conservation in those regions, but primarily focused on species richness and distribution in order to update scarce and fragmented historical records. A number of authors have reviewed freshwater fish conservation in individual med-regions, noting high rates of endemism and a severe threat of extinction in South Africa (Ellender et al., 2017), California (Moyle et al., 2013), and the Mediterranean basin (Hermoso & Clavero, 2011). Together, these papers demonstrate the precarious nature of freshwater biodiversity in med-regions; however, reviews spanning all med-regions are still required.

Freshwater mussels (Bivalvia, Unionida; hereafter 'mussels') are important functional components of aquatic ecosystems (Vaughn, 2010). They perform a variety of roles, including biofiltration, nutrient recycling and storage, provision of structural habitat, and modification of the sediment and surrounding food webs (Howard & Cuffey, 2006; Spooner & Vaughn, 2008; Atkinson, Kelly & Vaughn, 2014). Through these actions, mussels provide significant ecosystem services for humanity, and so there is concern over the consequences of the global decline of this group (Vaughn, 2018).

Mussels have a suite of biological characteristics that make them vulnerable to environmental change (Lydeard et al., 2004; Lopes-Lima et al., 2018b; Ferreira-Rodríguez et al., 2019). Adults are largely immobile, often with narrow environmental tolerances for salinity (Blakeslee et al., 2013; Klunzinger et al., 2015; Benson et al., 2019), desiccation (Gough, Gascho Landis & Stoeckel, 2012; Sousa et al., 2018a), and flow conditions (Sheldon & Walker, 1989; Strayer, 1999). Many mussel species are long-lived (sometimes >100 years), slow growing, and late to mature, so populations are unlikely to recover quickly from disturbance (Strayer et al., 2004; Benson et al., 2018; Benson et al., 2019). Larval mussels (glochidia) generally require a host fish for development and dispersal (Modesto et al., 2018), and the loss of suitable fish species and barriers to fish movement (e.g. dams and weirs) can eliminate mussel populations (Araujo & Ramos, 2000; Santos et al., 2015; Benson et al., 2018). Glochidia are particularly intolerant of environmental stressors, and populations may persist for years without adding new

recruits, creating an extinction debt (Haag, 2012; Ferreira-Rodríguez et al., 2019). The global decline of mussels has spurred research into various aspects of their taxonomy, biogeography, and ecology, in order to facilitate their conservation (Pereira et al., 2014; Walker, Jones & Klunzinger, 2014a; Lopes-Lima et al., 2017a; Lopes-Lima et al., 2017b; Zieritz et al., 2018; Brian & Aldridge, 2019).

Aspects of mussel conservation have been reviewed at various regional scales, including Europe (Lopes-Lima et al., 2017b), North America (Brian & Aldridge, 2019), East and Southeast Asia (Zieritz et al., 2018), and South America (Pereira et al., 2014). A recent global assessment showed that 41% of the described mussel species assessed by the IUCN were classified as Near Threatened, Vulnerable, Endangered, or Critically Endangered, whereas a further 17% were Data Deficient (Ferreira-Rodríguez et al., 2019; IUCN, 2019). Despite the role of climate in determining the hydrological characteristics that are an important driver of mussel distribution and abundance, to date there has been no review of mussel conservation within a specific climate type at a global scale. Many of the key threats to mussels are the same as those driving biodiversity declines in med-regions; however, there has been no synthesis of existing data on mussels that occur within med-region boundaries. In fact, a comprehensive catalogue of the mussel species in med-regions is yet to be compiled.

Given the alarming rate of mussel decline globally (Lydeard et al., 2004; Lopes-Lima et al., 2018b; Ferreira-Rodríguez et al., 2019), and the escalating threat to freshwater biodiversity in med-regions specifically (Bonada & Resh, 2013; Filipe, Lawrence & Bonada, 2013; Robson et al., 2013), the aim of this study was to assess med-mussel conservation relative to other mussel species. Specifically, three hypotheses were tested.

1. Med-mussels are more imperiled than other mussel species globally.
2. Med-mussels face a wider range of threats than other mussel species globally.
3. Med-mussels require greater conservation action than other mussel species globally.

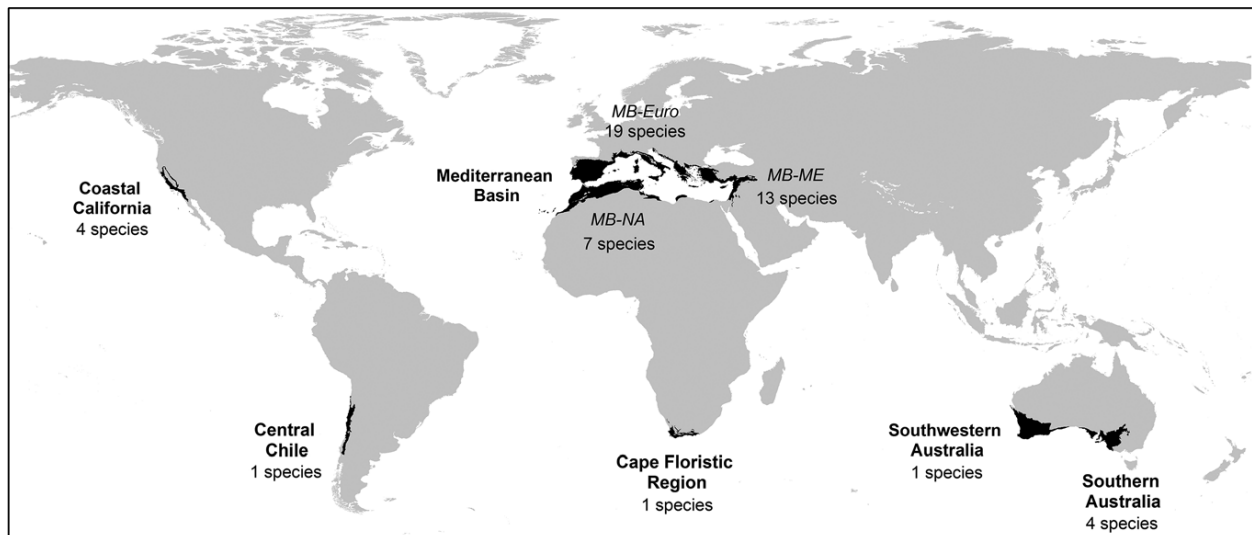
To test these hypotheses: (i) existing taxonomic literature was used to identify mussel species that occur within the boundaries of med-regions, and their geographical distribution; (ii) species richness and rates of endemism within each med-region were then assessed; and (iii) the IUCN Red List was reviewed to compile data on the conservation status, key threats, and conservation actions needed for mussels both within med-regions and globally. The findings are discussed in the context of the ecological implications for rivers and freshwater biodiversity conservation in med-regions.



## Methods

### *Med-region boundaries*

The geographical boundaries of med-regions were defined primarily using the Mediterranean forest, woodland, and scrub biome of the World Wildlife Fund Conservation Science Program (Olson et al., 2001). The Australian med-region can be divided into distinct southwestern (SWA) and southern (SA) subregions; these subregions are dealt with individually in this review, as they are separated by approximately 1,000 km of arid land and there is no overlap of mussel species between them (Bonada & Resh, 2013; Walker, Jones & Klunzinger, 2014a). Within the Mediterranean basin (MB), three subregions are defined to reflect differences in research effort in those areas (Bonada & Resh, 2013): European (MB-Euro), Middle Eastern (MB-ME), and North African (MB-NA) subregions. As a result, eight regions in total are defined in this article: four in the northern hemisphere – MB-Euro, MB-ME, MB-NA, and coastal California (CCali); and four in the southern hemisphere – SWA, SA, the Cape Floristic Region of South Africa (CFR), and Central Chile (CChile) (Figure 1).



**Figure 1.** Location of the global med-regions and mussel species richness per region. Regional boundaries are primarily based on the Mediterranean forest, woodland, and scrub biome of Olson et al. (2001). The Mediterranean basin (MB) is divided into European (MB-Euro), Middle Eastern (MB-ME), and North African (MB-NA) subregions. The MB-NA was limited to regions of Morocco, Algeria, and Tunisia falling within the ‘Permanent Maghreb’ region (based on Thieme et al., 2005) and coastal Libya. The Iberian Peninsula (MB-Euro), Syria, Lebanon, Palestine, Jordan, and Israel (MB-ME) are included in their entirety. The boundary between MB-Euro and MB-ME was defined as Istanbul, Turkey

### ***Species catalogue***

A species was defined as a med-mussel if all or part of its range fell within a med-region. A list of med-mussels was compiled in March 2019 using: (i) reviews on med-region freshwater biodiversity; (ii) reviews of the global/continental distribution of freshwater molluscs, bivalves, and mussels; (iii) the IUCN Red List of Threatened Species (<http://www.iucnRedList.org/>); and (iv) MUSSELP (<http://mussel-project.uwsp.edu>; Graf & Cummings, 2019) (references for individual species are supplied in Appendix 1). In order to account for recent taxonomic revisions the list was supplemented using relevant published literature, and such cases are discussed in the text.

### ***Conservation and threats***

The IUCN Red List was chosen as the tool for this review as it has been used to assess a large proportion of the mussel species known globally (approx. 67% as of 2017; Ferreira-Rodríguez et al., 2019). It provides a framework for evaluating species extinction risk and assigning conservation status based on population trends and geographical distribution (IUCN, 2019). Evaluated species are categorised as Extinct, Critically Endangered, Endangered, Vulnerable, Near Threatened, or Least Concern, or where there is insufficient information for accurate assessment, the species is listed as Data Deficient (IUCN, 2019). Species that have not been assessed are considered Not Evaluated. The term Imperiled is used to define species that are classified on the Red List as either Threatened (i.e. Critically Endangered, Endangered, or Vulnerable) or Near Threatened.

Evaluation on the IUCN Red List includes the identification of threats with impacts on the species. Threats are defined in a hierarchical classification scheme, which includes 12 broad threats (level 1), each comprising more specific threats at subsequent levels (levels 2 and 3). Red List evaluation also involves the recommendation of Conservation Actions Needed (CANs), which are defined in a similarly hierarchical classification scheme, with six level 1 actions, each comprising more specific actions at subsequent levels. Threats were reviewed at level 1 and CANs were reviewed at levels 1 and 2.

To quantify various aspects of med-mussel conservation, a list of all mussel species assessed with a global scope on the IUCN Red List was obtained in April 2019. Using the advanced search option of the IUCN's website, the 'Taxonomy' filter was applied (Animalia > Mollusca > Bivalvia > Unionoida), combined with filters for 'Red List Category', 'Threats', and 'Conservation Actions Needed'. The results were then divided into med- and non-med mussel species using the catalogue provided by this review. A number of med-species had been assessed with multiple scopes (e.g. globally, continentally, and

regionally). In these cases, the broadest scope was used for the analysis and the listings for smaller scopes are presented in Appendix 1.

A proportions test was used to compare the proportion of mussel species that are imperiled in med- and non-med regions. A quasi-Poisson generalised linear model was used to compare the number of level 1 threats and the number of level 1 CANs per species in med- and non-med regions. Quasi-Poisson models were chosen over Poisson models owing to evidence of overdispersion in the data ( $P \ll 0.0001$ , dispersion test). All statistical analysis was performed in *R* (R Core Team, 2017), with alpha set at 0.05.

## Results

### *Species richness*

Out of around 800 described mussel species globally (Ferreira-Rodríguez et al., 2019), 41 species are native to med-regions, with species richness highest in the MB (30 species; Appendix 1). Only four species are present in SA and CCali, and one species is present in CChile, in CFR, and in SWA. Despite the greater overall richness in the MB, most individual river basins in that region contain between one and four species only (Lopes-Lima et al., 2017b; Gomes-dos-Santos et al., 2019). Range declines in CCali have resulted in little overlap in the distribution of most species, such that there is often only a single species per basin where mussels still occur (Howard et al., 2015; Blevins et al., 2017). Similarly, only two species commonly occur in SA. Nineteen of the 41 med-mussel species are endemic to med-regions, with 17 of those occurring in the MB, whereas the other two species occur in Australia.

### *Taxonomy and distribution*

#### Mediterranean basin

Of the 30 mussel species occurring in the MB, 19 species occur in MB-Euro, seven species occur in MB-NA, and 13 species occur in MB-ME. Most species are restricted to just one of the three subregions, and only one species is known from all three subregions (Appendix 1). Recent taxonomic revisions have not only kept the exact number of species in flux (as is the case with mussels globally; Ferreira-Rodríguez et al., 2019), but have also altered the accepted geographical range of the revised species (Appendix 1). For example, the *Potomida* genus is currently considered monospecific, but has a long history of taxonomic revision and includes many polymorphic subspecies (Araujo et al., 2016; Froufe et al., 2016b). This review recognises three *Potomida* species, based on strong evidence that the genus should be divided into two distinct clades (Araujo et al., 2016; Froufe et al., 2016b), with one containing the widespread *Potomida littoralis* in MB-Euro, MB-NA, and beyond, and with the other containing two med-endemic

species, the Greek *Potomida acarnanica* (MB-Euro) and the Anatolan and Middle Eastern *Potomida semirugata* (MB-ME) (Froufe et al., 2016b).

There are 14 recognised morphospecies within the genus *Unio* in the Western Palaearctic; however, recent phylogenetic analysis suggests that *Unio* comprises at least 18–24 species (Araujo et al., 2017). All 14 morphospecies occur at least partly within the med-region, and this review recognises a 15th species, *Unio terminalis*, as it is currently listed separately from *Unio tigridis* in the IUCN Red List. There has been long-running uncertainty over the division of these two mussels as individual species. Although Araujo et al. (2017) recommended maintaining both under *U. tigridis* pending further investigation, they present evidence that samples from Israel and western Turkey were different species. *Unio tigridis* is found on the lower Orontes in Turkey and Syria, and in the Euphrates–Tigris basin, extending beyond the med-region boundary, possibly as far as Iran (Lopes-Lima & Seddon, 2014d). *Unio terminalis* appears to be endemic to MB-ME, having been recorded from the Orontes and Jordan River basins, and from coastal rivers in Syria, Lebanon, and Israel. *Unio terminalis* is likely to have disappeared from much of its range because of habitat degradation, and a subspecies (*Unio terminalis delicatus*) is considered extinct in Israel (Milstein, Mienis & Rittner, 2012).

*Unio bruguierianus* and *Unio ionicus* were recently confirmed as independent from the *Unio crassus* species complex based on genetic analysis (Araujo et al., 2017). Both species appear to be endemic to MB-Euro, with *Unio bruguierianus* only known from eastern Greece and with *Unio ionicus* only known from western Greece and Albania (Araujo et al., 2017). The *U. crassus* complex is widespread throughout Europe and into the Middle East, including rivers and lakes in med-regions. It contains many subspecies, some of which may also prove to be unique species or to be more appropriately placed within the complex of a congener (Lopes-Lima et al., 2017b). Araujo et al. (2017) suggested that the remainder of the *U. crassus* species complex (i.e. in addition to *U. bruguierianus* and *U. ionicus*) could be divided into as many as six lineages, and that three other *Unio* species (*Unio gibbus*, *Unio pictorum*, and *Unio ravoisieri*) could also be split into at least two lineages each; however, revisions have not been published at this point.

Historically, most MB-NA species have been considered to have close affinities with the Iberian Peninsula in MB-Euro. For example, *U. gibbus* is endemic to MB-Euro and MB-NA, and populations from Morocco are more closely aligned with those from Spain than with those in Tunisia (Araujo et al., 2017). *Unio delphinus* was, until recently, thought to occur in both Iberia (MB-Euro) and Morocco (MB-NA) until IUCN assessments recognised the African form as a unique species, *Unio foucauldianus* (Van Damme &

Ghamizi, 2010c; Araujo, 2011a). This division was later supported by phylogenetic analysis showing two geographically concordant clades: *U. delphinus* endemic to the western half of Iberia and *U. foucauldianus* with a widespread although patchy distribution in Morocco (Froufe et al., 2016a).

Five species from the genus *Anodonta* are known to occur within the MB (Appendix 1), including a newly described species, *Anodonta exulcerata*, which is commonly occurring and endemic to the Italian Peninsula and the Croatian Adriatic coast (Froufe et al., 2017). *Anodonta anatina* and *Anodonta cygnea* are both non-endemic and very wide-ranging, particularly in Europe, and both are found in MB-Euro and MB-ME (Smith et al., 2014; Lopes-Lima et al., 2017b). *Anodonta anatina* has also been confirmed genetically in MB-NA (Gomes-dos-Santos et al., 2019), whereas reports of *A. cygnea* from Algeria require similar confirmation (Lopes-Lima, 2014c). The two remaining *Anodonta* species, *Anodonta pseudodopsis* and *Anodonta vescoiana*, are found in MB-ME, where *A. pseudodopsis* is restricted to the Orontes basin, but where the distribution of *A. vescoiana* extends beyond the med-region into Iraq (Lopes-Lima & Seddon, 2014a; Lopes-Lima, 2014b).

The family Margaritiferidae has a complicated taxonomic and phylogenetic history, including a wide-ranging and disjunct distribution in North America, Eurasia, and North Africa (Bolotov et al., 2016). The systematics of the family was recently redefined by Lopes-Lima et al. (2018a). Three of the four margaritiferid species in the MB were reassigned to the genus *Pseudunio* (*Margaritifera auricularia* to *Pseudunio auricularius*, *Margaritifera homsensis* to *Pseudunio homsensis*, and *Margaritifera marocana* to *Pseudunio marocanus*). *Pseudunio* is used here, but all remain Red Listed under the genus *Margaritifera*. *Pseudunio auricularius* was once widespread throughout Europe, including the majority of MB-Euro. It was thought to be extinct until being rediscovered in 1985, and is currently known from four catchments in France outside of the med-region and from one river on the Iberian Peninsula in MB-Euro (Prié et al., 2018). *Pseudunio homsensis* is endemic to the Orontes basin and possibly other MB-ME river systems nearby; however, it was only known from museum collections until one living individual and other evidence of an extant population (i.e. shell fragments) were discovered in the Karasu River (Vikhrev et al., 2018). *Pseudunio marocanus* is endemic to MB-NA and is considered the rarest bivalve in African fresh waters (Sousa et al., 2016). Its distribution and abundance have declined dramatically in the past century, and it is now constrained to just a few locations in Morocco (Sousa et al., 2018b). The fourth margaritiferid species, *Margaritifera margaritifera* is a Holarctic species, ranging from North America to Europe and into Siberia. It is considered Endangered globally (Moorkens et al., 2018) and

Critically Endangered within its European range (Moorkens, 2011). Within MB-Euro, it is only known to occur in the northern Iberian Peninsula, generally at very low abundances (Sousa et al., 2015).

#### Coastal California

There are four recognised species within CCali: *Anodonta californiensis*, *Anodonta nutalliana*, *Margaritifera falcata*, and *Gonidiea angulata* (Ball et al., 2013; Williams et al., 2017). The boundary of CCali falls within the Pacific region of North America, which has a mussel fauna that is evolutionarily distinct from the remainder of the continent (Haag, 2010); however, none of the four species is endemic to the med-region. All four species have a long history of taxonomic revision: the two *Anodonta* species are often considered synonymous under *A. nutalliana* (Mock et al., 2010), and are currently listed as such on the IUCN Red List (Blevins et al., 2016a). Based on Williams et al. (2017), both *Anodonta* species are recognised in this review, but they are discussed together owing to the difficulty of disentangling their individual distributions. Although there are historical records of additional *Anodonta* species in the region (*Anodonta oregonensis* and *Anodonta kennerlyi*), recent studies suggest that these are erroneous (Mock et al., 2010; Blevins et al., 2017). Museum records and published literature show that mussels once occurred throughout California, in many cases being highly abundant (Howard et al., 2015). Recent surveys have found that they have declined substantially, and appear to have been extirpated in much of CCali (Howard et al., 2015; Blevins et al., 2017).

Historical collections and observations indicate that *A. californiensis* and *A. nutalliana* inhabited many hydrological basins west of the Rocky Mountains, from Canada to northern Mexico (Mock et al., 2010). Within the coastal boundary of CCali, populations were known as far south as San Diego according to the mapped distribution on the IUCN Red List (Blevins et al., 2016a). Based on morphology, the two species co-occur; however, individuals of the two morphotypes from the same river are genetically more similar to one another than to individuals with similar morphology from other rivers (Mock et al., 2010), highlighting the difficulty in assigning species-level classification to mussels using morphology alone. *Anodonta nutalliana* (including the synonymous *A. californiensis*) appears to have been extirpated from most of southern California (Howard et al., 2015; Blevins et al., 2017).

*Margaritifera falcata* is the widest ranging of the med-mussels in the region, with populations found across the continental divide and into the headwaters of the Missouri (Gangloff & Gustafson, 2000). On the west coast, the species ranges from Canada south into the med-region, as far as the Kern River basin near Los Angeles. The species extent of occurrence has declined only slightly, but this greatly

underestimates the declines in catchment area and abundance (Blevins et al., 2017). It is the only mussel to have been found in southern California between 1990 and 2015 (Howard et al., 2015).

The distribution of *Gonidea angulata* is believed to have always been much smaller than the distributions of the other CCali species, although historical records show that it ranged from Canada to the Santa Ana River basin south of Los Angeles (Blevins et al., 2016b). It appears to have been lost from much of its former range and most extant populations are now found north of the boundary of the med-region (Howard et al., 2015). It is still known from locations as far south as Napa County near San Francisco (Blevins et al., 2017).

#### Central Chile

*Diplodon chilensis* is the only mussel found in CChile, where it is common and often abundant (Lara & Parada, 2009). It is also widespread outside of the med-region, occurring in all five hydrographical zones of Chile as well as in parts of Argentina (Fuatealba, Figueroa & Morrone, 2010). Recently, it has declined in abundance in parts of its range, including in the med-region, and has been extirpated from some locations (Valdovinos & Pedreros, 2007). A second Chilean mussel species, *Diplodon solidulus*, is not considered here because it is only found at Puerto Montt, just south of the boundary of CChile (Fuatealba, Figueroa & Morrone, 2010).

#### Cape Floristic Region

*Unio caffer* (syn. *Cafferia caffra*) is the only mussel species found in the CFR, and has a distribution that is highly disjunct from the remainder of the *Unio* genus (Lopes-Lima et al., 2017a). It is widespread and common throughout the southern part of the African continent; however, knowledge of its historical distribution within the CFR and South Africa in general is limited (de Kock & Wolmarans, 2010; Graf & Cummings, 2011). A recent review of museum records shows that *U. caffer* was more widely distributed within South Africa than previously reported (Sonamzi et al., 2019). The CFR constitutes only a small part of the overall range, but the observed decline there has been dramatic (Day & de Moor, 2002) and there do not appear to be any examples of extant populations in recently published literature.

#### Southwestern Australia

*Westralunio carteri* is the only mussel in SWA, where it is widespread and endemic (Walker, Jones & Klunzinger, 2014a). Recent phylogeographical work found support for the separation of *W. carteri* into distinct western and southern lineages, with additional substructuring within the southern lineage (Klunzinger et al., 2020). The extent of occurrence for *W. carteri* is estimated to have declined by 49% in

the last three generations, and it now appears to be restricted to freshwater lakes, rivers, and streams, within 50–100 km of the coast (Klunzinger et al., 2015). Where they still occur, population densities can be high (with  $\geq 20$ –50 mussels/m<sup>2</sup>; Benson et al., 2018; Ma, 2018). It is a highly disjunct member of its genus, with the other two congeners found in New Guinea (Walker, Jones & Klunzinger, 2014a).

#### Southern Australia

Four mussel species occur within SA (*Alathyria jacksoni*, *Hyridella glenelgensis*, *Hyridella narracanensis*, and *Velesunio ambiguus*), and it is the only southern hemisphere med-region with multiple species. *Alathyria jacksoni* and *V. ambiguus* are wide ranging in the eastern states of Australia, with most of their distribution falling outside of the med-region (Jones & Byrne, 2014; Walker, Jones & Klunzinger, 2014a). Both species have shown some evidence of decline within the med-region, and *V. ambiguus* has been lost from parts of the lower Murray River in SA as a result of increasing salinity caused by evapoconcentration (Kingsford et al., 2011; Walker, 2017).

*Hyridella narracanensis* is rare and sparsely distributed in coastal southeastern Australia, and it is only known from one location within the med-region, whereas *H. glenelgensis* is endemic to a single freshwater tributary of the Glenelg River in western Victoria (Playford & Walker, 2008; Jones & Byrne, 2014). Preliminary genetic evidence suggests that *H. glenelgensis* and *H. narracanensis* are morphological variants of one species (Playford & Walker, 2008).

#### **Conservation status**

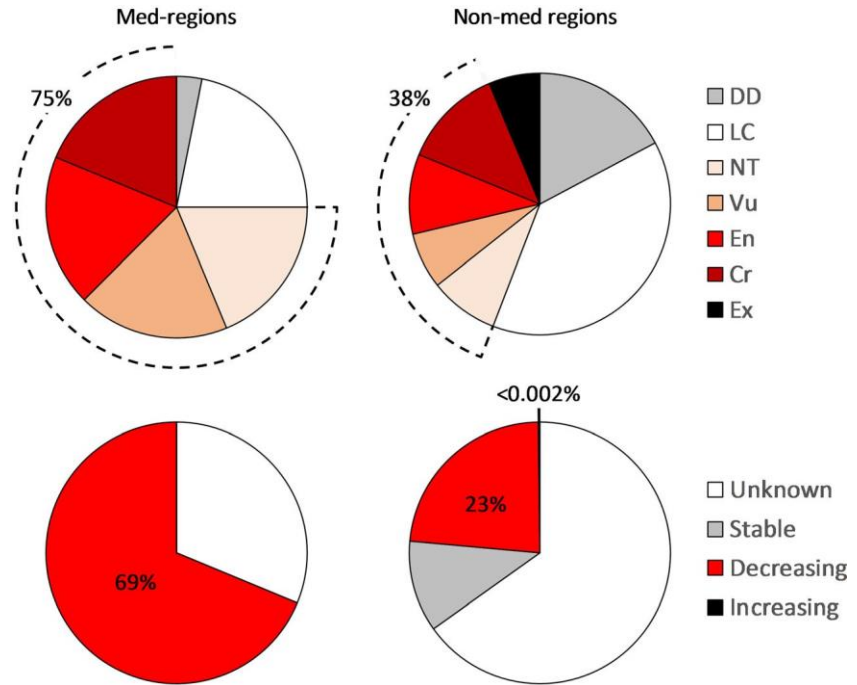
Of the 41 med-mussel species, 32 (78.0%) have been assessed compared with 505 (approx. 66.5%) non-med species. The MB accounts for 22 (68.8%) of the assessed med-mussels, although a number of recently recognised species in that region now require assessment. Only one med-mussel species (3.1% of those assessed) was listed as Data Deficient, compared with 87 (17.2%) for non-med species. Only one non-endemic species, *U. crassus*, has received an evaluation specific to its med-region distribution, where it is listed as Data Deficient, while being listed as Endangered globally and Vulnerable within Europe (Appendix 1).

Med-mussels are almost twice as likely to be Imperiled as non-med mussels (75% compared with 38% of species;  $\chi^2 = 15.812$ ;  $P < 0.0001$ ; Figure 2). No med-mussels are listed as Extinct; however, reports of extant populations are rare for some species (e.g. *Leguminaia saulcyi* and *Pseudunio homsensis*; Vikhrev et al., 2018). The population trend for all med-mussels is either Decreasing or Unknown, whereas only



70% of non-med species fall within these categories (Figure 2). All 13 of the listed med-mussels that are endemic to a med-region are considered to be Imperiled.

**Figure 2.** Comparison of IUCN Red List information for freshwater mussels in med-regions (left;  $n = 32$ ) and non-med regions ( $n = 505$ ) in terms of conservation status (top) and population trend (bottom). The dashed lines indicate the proportions of Imperiled, Threatened (i.e. Critically Endangered, Endangered, or Vulnerable), and Near Threatened mussels



Comparing med-regions, 78.3% of mussels from the MB are considered to be Imperiled compared with 66.7% for all other med-mussels, and five of the six Critically Endangered med-mussels occur in the MB. In CCal, the three listed species (*A. nuttalliana*, *G. angulata*, and *M. falcata*) are all Imperiled. The fourth species, *A. californiensis*, does not have its own assessment as it is considered a synonym of *A. nuttalliana* on the Red List. In the southern hemisphere, the conservation status of med-mussel species strongly reflects their wide distributions and lack of endemism, and only three of seven species are Imperiled. Of those, *H. glenelgensis* (SA) is concurrently the only narrow-range endemic outside of the MB and the only Critically Endangered species outside of that region. *Hyridella narracanensis* (also SA) is non-endemic, but its distribution is relatively narrow and highly fragmented (Jones & Byrne, 2014). It was recently upgraded from Data Deficient to Near Threatened; however, its extent of occurrence only slightly exceeds the cut-off for being listed as Vulnerable (Klunzinger, Walker & Jones, 2014). The third Imperiled species from the southern hemisphere, *W. carteri*, is endemic to SWA but is widespread near

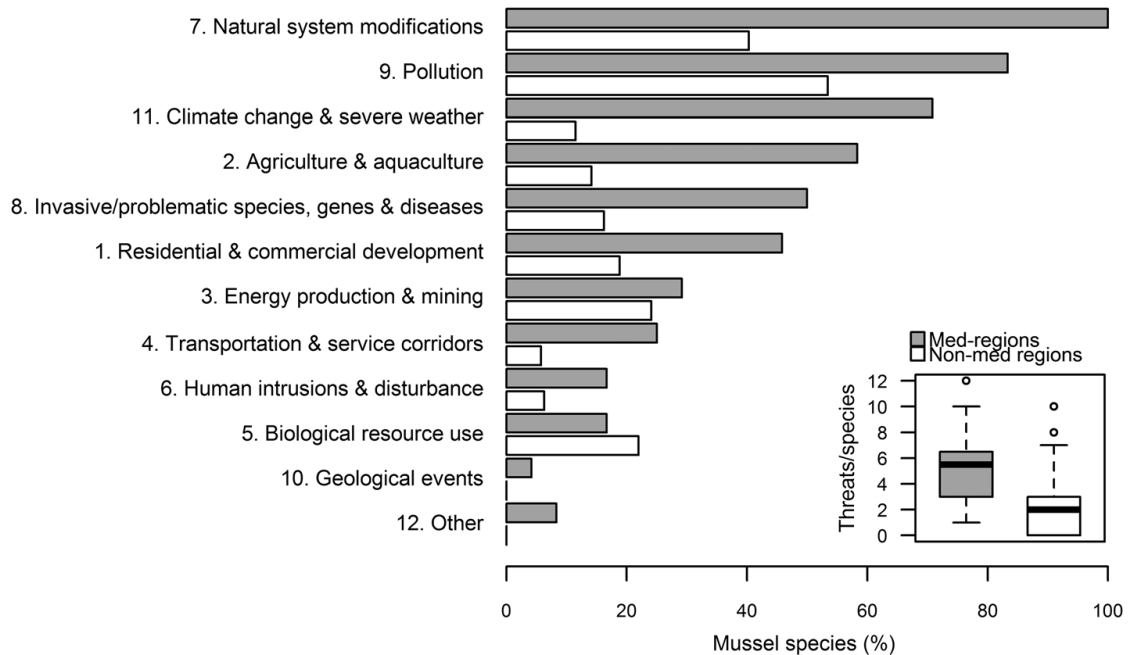
the coast in that region. Of the non-Imperiled species in the southern hemisphere, *D. chilensis* (CChile) and *U. caffer* (CFR) are listed as Least Concern despite strong evidence of decline for populations within their med-regions, as both species have wide-ranging distributions. Similarly, *A. jacksoni* and *V. ambiguus* (both from SA) are listed as Data Deficient and Not Evaluated, respectively, and are widely distributed in eastern Australia.

### **Key threats to Imperiled mussels**

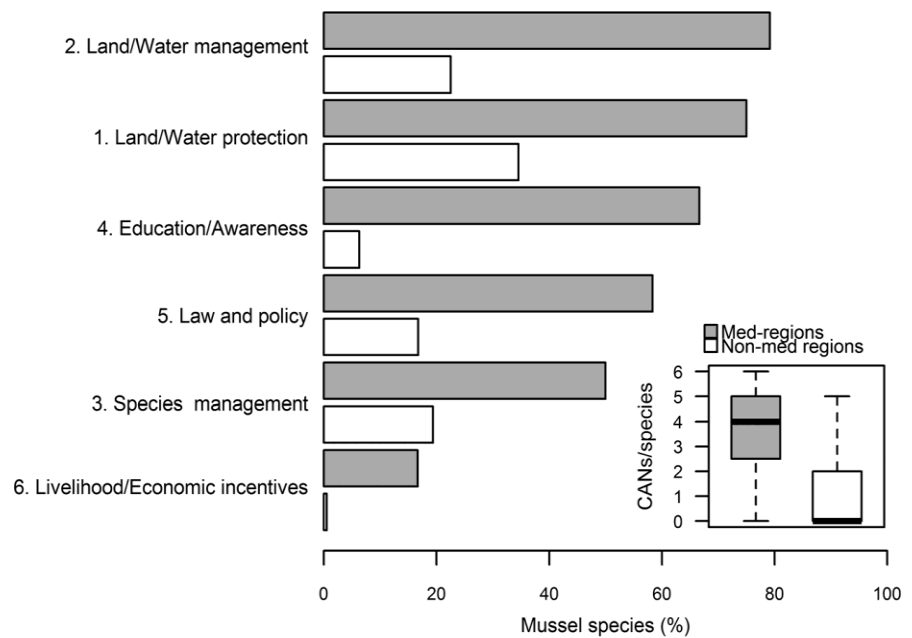
As a group, Imperiled med-mussel species are subjected to a wider range of threats than Imperiled non-med mussel species (2.4 times more threats per species;  $t = -5.521$ ;  $P << 0.0001$ ; Figure 3): 25% of med-species are subject to six or more threats, compared with just 3.7% for non-med species, and similarly, 8% of med-species are subject to 10 or more threats, compared with just 1.6% of non-med species.

Individually, 11 of the 12 threats affect a higher proportion of Imperiled med-mussel species than non-med mussel species (Figure 3). Med-mussels are particularly affected by 'Natural systems modifications' (100% of all species), 'Pollution' (83%), 'Climate change and severe weather' (71%), and 'Agriculture and aquaculture' (58%) (Figure 3). For the non-med species, the most common threat is 'Pollution' (53% of all species). Seven of the 12 threats are more than twice as prevalent among med-mussels than among non-med mussels, with the differences particularly noticeable for 'Climate change and severe weather' (>6×), 'Transportation and service corridors' (>4×), 'Agriculture and aquaculture' (>4×), and 'Invasive/problematic species, genes, and diseases' (>3×) (Figure 3). 'Biological resource use' is the only threat that is less common for med-mussel species.

Comparing among med-regions, MB mussels are affected by 4.3 threats per species, whereas CCali mussels are affected by six threats per species. Australian species are generally subject to a wider range of threats than other med-mussels: *H. glenelgensis*, seven threats; *H. narracanensis*, eight threats; and *W. carteri*, 12 threats. The only other med-mussels subject to more than six threats are the MB species *M. margaritifera* (10 threats), *P. auricularius* (seven threats), and *P. maroccanus* (seven threats).



**Figure 3.** Comparison of level-1 threats to Imperiled freshwater mussels in med-regions ( $n = 24$ ) and non-med regions ( $n = 191$ ), based on the IUCN Red List. The term Imperiled includes species listed as Threatened (i.e. Critically Endangered, Endangered, or Vulnerable) and Near Threatened. The box plot shows the number of threats per species. The bar plot shows the percentage of species subject to each of the 12 level-1 threats

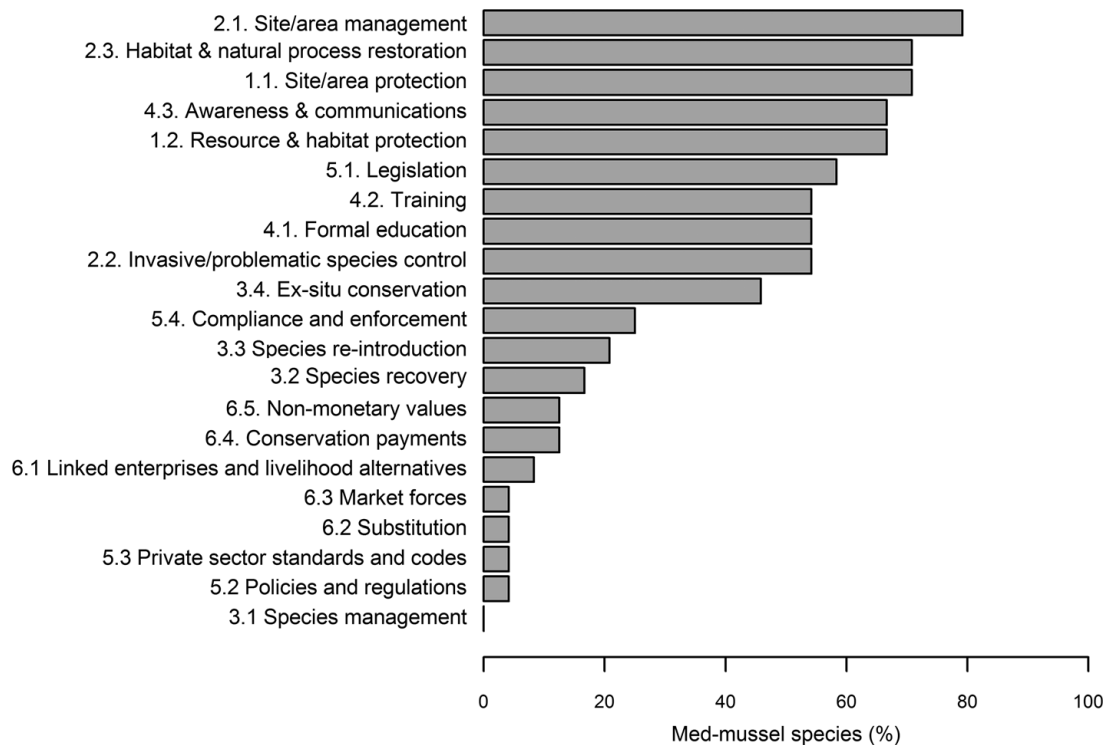


**Figure 4.** Comparison of level-1 Conservation Actions Needed (CANs) recommended for Imperiled freshwater mussels in med-regions ( $n = 24$ ) and non-med regions ( $n = 191$ ), based on the IUCN Red List. The term Imperiled includes species listed as Threatened (i.e. Critically Endangered, Endangered, or Vulnerable) and Near Threatened. The box plot shows the number of CANs recommended per species. The bar plot shows the percentage of species requiring each of the six level-1 CANs

### Conservation actions needed for Imperiled mussels

Imperiled med-mussel species require 3.5 times more level 1 CANs per species than non-med mussel species ( $t = -6.683$ ;  $P < 0.0001$ ; Figure 4). Only four species globally require all six level 1 CANs, and all of those are from med-regions: three from Australia (*H. glenelgensis*, *H. narracanensis*, and *W. carteri*) and one from the MB (*M. margaritifera*). No CANs have been recommended for the three listed CCali species.

Each of the six level 1 CANs are recommended at least twice as often for med-mussel species as they are for non-med mussel species (Figure 4). The most common level 1 CANs for med-mussels are 'Land/Water management' and 'Land/Water protection' (Figure 4), and all level 2 actions within those CANs are required by more than 50% of med-species (Figure 5). 'Species management' and 'Livelihood and economic incentives' are the only level 1 CANs recommended for 50% or fewer med-species. In contrast, 'Land/Water protection' is the most common level 1 CAN for non-med mussels, being recommended for 35% of species (Figure 4).



**Figure 5.** The percentage of Imperiled, including Threatened (i.e. Critically Endangered, Endangered, or Vulnerable) and Near Threatened, med- mussel species ( $n = 24$ ) requiring each of the IUCNs 21 level-2 Conservation Actions Needed (CANs)

## Discussion

This review identified 41 species of mussels that occur in med-regions. Med-mussel assemblages in many med-rivers are characterised by low species richness, often with just a single species. Med-mussels species are more imperiled, face a wider range of threats, and require more extensive conservation actions in order to prevent extinction than non-med mussels. All species endemic to med-regions are imperiled, and although the extinction risk is lower for non-endemic species, most have declined within the boundary of their med-region, including all such species in the southern hemisphere. This suggests that all mussel populations within med-regions require greater conservation effort; however, only one non-endemic species has been assessed specifically within its med-region (*U. crassus*, which is Data Deficient in the Mediterranean basin). All med-mussels are threatened by 'Natural systems modification', and conservation actions focus on the management and protection of these areas.

There are four key implications of these findings. First, low mussel species richness means that there is often no redundancy within this group, and in these cases the loss of a mussel population would result in the loss of all mussel-derived ecosystem services. Second, current Red List status is likely to underestimate the degree of imperilment for populations of non-endemic species in med-regions. Third, many rivers in med-regions have been highly modified through actions such as channel diversions and the construction of dams and weirs (Bonada & Resh, 2013). In some cases, such modifications may create novel freshwater habitats and refuges capable of bolstering mussel conservation. Finally, much of the research focus in med-regions is on the MB, and the decline in mussel species in other med-regions may continue unabated without a concerted effort to conserve populations in these areas.

### *Low species richness means less functional redundancy*

Mussels are described as ecosystem engineers as they perform a variety of important functional roles, including water purification, nutrient cycling, and sediment restructuring (reviewed in Vaughn & Hakenkamp, 2001). Med-regions support far fewer mussel species (generally with between one and four per river basin) than other places, such as most of non-med Europe (generally with between five and 10 species per river basin; Lopes-Lima et al., 2017b), east and Southeast Asia (often with between six and 68 species per river basin; Zieritz et al., 2018), and eastern North America (with as many as 104 species per river basin; Haag, 2012). Despite the low level of richness in med-regions, some species are known to have been abundant historically (Bogan & Cummings, 2011; Klunzinger & Walker, 2014). Although the importance of research conducted in hot spots of mussel species richness is undoubted, the loss of

these ecosystem engineers might be more severely felt in regions where species richness in the group is low, and the conservation of such species requires immediate attention.

The resilience of ecosystems to disturbance relies on the diversity of responses from species performing the same functional role ('response diversity'; Elmqvist et al., 2003; Mori, Lertzman & Gustafsson, 2017). In a diverse group, some species may actually respond positively to environmental change, and there is a degree of functional redundancy and 'insurance' if one species is lost (Yachi & Loreau, 1999; Loreau et al., 2001). Many mussel species globally are large and long-lived, often congregating in dense, multi-species beds (Haag, 2012), and species decline can significantly alter ecosystem functioning and associated ecosystem services (Spooner & Vaughn, 2008; Vaughn, 2010). For many freshwater ecosystems in med-regions, the extinction of one mussel species would result in the complete loss of all mussel-derived ecosystem services.

There have been few studies on ecosystem engineering by mussels in med-regions. In CChile, filter feeding by *D. chilensis* has been estimated to turn over the volume of Laguna Chica de San Pedro more than 80 times per year (Fuentealba & Henriquez, 2009), and may be instrumental in preventing eutrophication from salmon farming in Chilean lakes (Soto & Mena, 1999). Similarly, biodeposition by *M. falcata* increases benthic resources, leading to increased macroinvertebrate biomass in CCali river sediment (Howard & Cuffey, 2006). 'Habitat and natural process restoration' is among the most commonly recommended conservation actions for med-mussels; however, it should be recognised that mussels are important both as habitat and for processes required by other organisms.

#### *The imperilment of mussels in med-regions is underestimated*

The results of this review show that med-mussels are more imperiled than non-med mussels; however, a number of factors suggest that the level of imperilment may be underestimated. First, all of the 13 species endemic to med-regions that have been evaluated are imperiled. Although for most of those species this reflects a restricted distribution, some have wider ranges and yet are still imperiled (e.g. *W. carteri*, SWA; *Microcondylaea bonellii*, MB-Euro). Second, accurate Red List evaluation requires knowledge of the threats faced; however, specific threats are at present unknown for many endemic species (e.g. *A. pseudodopsis*, *A. vescoiana*, *L. saulcyi*, and *P. homsensis*). Third, in these cases the named threats are those known to affect mussel species and freshwater systems in med-regions generally (e.g. 'Natural system modification', 'Pollution', and 'Climate change and severe weather'), so populations of non-endemic species are also likely to be susceptible. Only 11/19 non-endemic med-mussel species are

currently considered Imperiled; however, only one has been assessed specifically within its med-region (Appendix 1). There is at present a lack of resolution in the assessment of non-endemic med-mussels that prevents the adequate conservation of populations within med-regions.

Regional assessments would be valuable for many non-endemic med-mussels. For example, *U. caffer* (Least Concern) has experienced an unexplained and dramatic decline within the CFR, a number of populations of *D. chilensis* (Least Concern) have been lost or are considered 'terminal' in CChile (Valdovinos & Pedreros, 2007), and three of the four CCali species have not been detected in southern California since 1995 (Howard et al., 2015). The extinction risk for wide-ranging species is lessened when genes and individuals can be replenished by neighbouring populations; however, all med-regions are bordered on one side by an ocean, and the adjoining landscapes are often arid or cut off by large mountain ranges. Many rivers in med-regions are short and fall completely within the med-region boundary (e.g. CFR and SWA), limiting the dispersal potential for riverine fauna, which is already restricted owing to the discrete nature of river basins. Connectivity in med-rivers is further reduced by the prevalence of intermittent systems, which are becoming more common as a result of climate change, natural system modifications, and resource exploitation (Cooper et al., 2013; Robson et al., 2013; Gomes-dos-Santos et al., 2019). Mussel dispersal is largely determined by the availability and mobility of glochidial hosts, but many freshwater fishes in med-regions are themselves highly imperiled (Ellender et al., 2017). Although there are some recent examples of natural recolonisation by med-mussels, these have been at scales of hundreds of metres, and recovery over larger distances is yet to be observed (Benson et al., 2018; Benson et al., 2019). In combination, these factors indicate that all mussel populations in med-regions may be more imperiled than current assessments suggest.

#### *Identification of 'novel' habitats and refuges*

This review showed that 'Natural system modification' is the most common threat to med-mussels. As modified systems become increasingly common in med-regions, the identification of novel systems capable of providing additional habitat could bolster conservation efforts. A number of recent studies have shown that novel habitats can provide refuge for mussels and other freshwater organisms in med-regions, particularly in response to threats posed by climate change. Water-mill canals in MB-Euro can support populations of *M. margaritifera* if water levels are appropriately managed during extreme droughts (Sousa et al., 2019a). Similarly, *P. marocanus* was found to have successfully colonised an irrigation canal in MB-NA; a lack of mussels in a neighbouring canal was attributed to frequent dredging and cleaning activities (Sousa et al., 2019b). These examples highlight that the efficacy of novel systems

for conservation will depend on an understanding of their unique characteristics and management needs. Without such knowledge, otherwise suitable habitats might in fact act as ‘traps’, leading to reduced survival or reproduction (sensu Schlaepfer, Runge & Sherman, 2002).

Understanding the critical features of existing novel habitats could also allow the identification of other potential habitats within the natural range of a species. For example, many catchments in the salinised landscape of SWA are scattered with artificial water points, constructed for agriculture and fire management (Beatty et al., 2017). These water points might act as freshwater refuges around salinised rivers and provide a source population for future recolonisation in river systems should salinity be reversed (sensu Benson et al., 2019). Mussels are known to occur in these habitats (<https://rivers.dwer.wa.gov.au/species/westralunio-carteri/>); however, it is not known whether colonisation occurred naturally or was assisted, or whether the populations found there are self-sustaining. More research in this area is needed.

#### *Research effort among med-regions is uneven*

The large number of species in the MB, relative to other med-regions, probably results from its much larger geographical area at the intersection of three major land masses, but also reflects a greater level of taxonomic research in that region. Such research has not only refined regional taxonomy, but also informs conservation decision making. Other med-regions may also host cryptic species (Klunzinger et al., 2020), but research in this field is generally lacking. The CFR, for example, typically has short coastal rivers that are geographically isolated, resulting in exceptional levels of regional endemism in freshwater fauna (Darwall et al., 2009; Ellender et al., 2017). Many fish in that region are narrow-range endemics, restricted to single river systems or even tributaries (Ellender et al., 2017). It seems likely that *U. caffer* populations might be similarly distinct ecological units, but this has not been assessed.

Additional population genetic work could identify evolutionarily significant populations or management units for those med-regions not yet assessed. In the MB-NA, Gomes-dos-Santos et al. (2019) found that mussel assemblages had greater species richness, genetic diversity, and range size in perennial rivers near the coast than in intermittent rivers in the more arid inland areas. Identifying such features is valuable for conservation efforts, because populations with greater genetic diversity may be more robust and have a greater ‘response diversity’ to disturbance. Alternatively, populations from hotter and drier areas may be arid-adapted, and better able to tolerate projected climate scenarios (Gomes-dos-Santos et al., 2019). Differences in species richness and genetic diversity in mussel assemblages from



arid areas may also provide a window into the prospects for med-mussels facing climate change. At present, little is known about genetic structure within and among populations of most med-mussel species, particularly in the southern hemisphere.

Most med-regions are lacking up-to-date information on species distribution and basic demographic data that are essential for accurate conservation planning. For example, whereas a recent review of museum records updated the historical distribution of *U. caffer* within South Africa (Sonamzi et al., 2019), information on the present situation of those populations is lacking. Similarly, *D. chilensis* is considered to be the best-known member of its family (Hyriidae) in South America (Pereira et al., 2014), yet many rivers in CChile remain unexplored (Figueroa et al., 2013). The distribution of *W. carteri* in SWA was recently updated, including the discovery of new populations (Klunzinger et al., 2012; Klunzinger et al., 2015), but population densities and size structure are only known for a few locations (Benson et al., 2018; Ma, 2018; Benson et al., 2019). In the MB, the recent division of some species complexes into multiple species has added greatly to our knowledge of mussel distribution in that region. Red List evaluations are now needed for the 'new' species, and the evaluation of the existing species requires updating. Understanding species distribution and abundance is fundamental to ecology and the current lack of knowledge on this and other topics limits the effective conservation of med-mussel species.

#### *Future research directions*

Mussels are both globally imperiled and ecologically important. Understanding the nature of the threatening processes driving their decline, and how to mitigate those threats in med-regions, and other climate regions, will benefit freshwater ecosystems. Although most med-mussel species have been assessed under the IUCN criteria, opportunities to refine aspects of their conservation remain. In many cases there is limited understanding of the basic ecological requirements and exact threats faced by a species. Non-endemic med-mussels require focused regional assessments, as the level of imperilment might be greater for populations within med-regions than for the species as a whole. Research targeting the major threats identified here (e.g. natural systems modification, pollution, and climate change) will help to mitigate them and to identify opportunities for conservation (e.g. canals and artificial water points). More research on these topics is needed, particularly for species in the southern hemisphere, if med-mussels are to be appropriately managed and conserved.

It is important to note that many non-med mussel species are also imperiled. Additional reviews of species richness and conservation in other climate types would benefit global mussel conservation. Such

reviews could identify other areas experiencing elevated levels of threat and facilitate comparison among regions. Our article demonstrates the complexity of compiling a comprehensive regional species catalogue at a global scale. Species boundaries for mussels have been notoriously hard to define owing to a history of taxonomic revisions and the morphological convergence of co-occurring species (Walker, Jones & Klunzinger, 2014a; Williams et al., 2017; Lopes-Lima et al., 2017b). Advances in molecular methods and greater accessibility to these tools are helping to disentangle species distributions (Mock et al., 2010; Froufe et al., 2016b), and must be accounted for in any review. This information will inform pre-emptive species conservation, especially in regions where climate shifts are expected to be pronounced.

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## Chapter 3:

### Upstream recolonisation by freshwater mussels (Unionoida: Hyriidae) following installation of a fishway



**Cover picture:** 'Evidence' of a mussel hitchhiking up the Goodga fishway on *Galaxias truttaceus*

Benson, J.A., Close, P.G., Stewart, B.A. & Lymbery, A. (2018). Upstream recolonisation by freshwater mussels (Unionoida: Hyriidae) following installation of a fishway. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 28(2), 512-517. <https://doi.org/10.1002/aqc.2861>

## Abstract

Freshwater mussels provide important benefits to aquatic ecosystems by filtering water, bioturbating sediments, and cycling and transforming nutrients. The global decline in mussel diversity, distribution and abundance has led to concerns that ecological functioning in freshwater systems will be diminished. Mussels from the order Unionoida have an obligate larval stage that parasitises a fish host, developing into a juvenile while being dispersed throughout the ecosystem. Barriers that obstruct fish movement can lead to localised extinctions of fish and mussels. In many cases, fishways have successfully restored habitat connectivity for fish; however, mussel recolonisation is rarely assessed. This paper provides evidence for recolonisation by Carter's freshwater mussel (*Westralunio carteri*, Iredale 1934) in habitats upstream of a weir following fishway installation. Mussels were present at all sites both above and below the weir, although they were far more abundant downstream. A lack of larger size classes upstream highlights the historical lack of recruitment in that area. Recent recruitment post-fishway installation suggests that the population will eventually recover above the weir. The return of mussels above the weir is likely to benefit the ecosystem owing to the key role mussels play in freshwater habitats. Fishways may therefore be an important tool for the restoration of mussels, and broader ecological functioning.

## Introduction

Longitudinal connectivity within rivers is essential to the ecology of fish and other taxa, and integral to ecosystem functioning (Dudgeon et al., 2006). Instream barriers such as dams and weirs impair animal movements and are a major cause of decreased species abundance and diversity in freshwater ecosystems (Rolls et al., 2013). The removal of barriers is increasingly undertaken in many countries as a part of stream restoration projects; however, removal is not always possible where the function of the barrier is still required (Gangloff, 2013). Although the construction of 'fishways' (sometimes termed fish ladders) has been shown to restore connectivity for fish in many cases, many fishways do not provide adequate passage for all freshwater fauna (Harris et al., 2017), and the benefits to broader ecosystem functioning are rarely assessed.

One taxon that is expected to benefit from fishways is Unionoida (freshwater mussels; hereafter 'mussels'). Mussels have an obligate larval stage (described as either a glochidium or lasidium, depending on higher taxonomic classification (Walker, Jones, & Klunzinger, 2014)) that parasitises a fish host, during which time they mature into a juvenile mussel and are dispersed throughout the ecosystem (Arvidsson, Karlsson, & Österling, 2012; Strayer et al., 2004; Watters, 1996). Barriers that restrict fish movement also interfere with mussel recruitment, and therefore distribution and abundance (Brainwood, Burgin, & Byrne, 2008; Strayer et al., 2004; Watters, 1996), and are a major contributor to the global decline of mussels (Araujo & Álvarez-Cobelas, 2016; Lydeard et al., 2004). Although a number of authors suggest that fishways will aid the restoration of mussel populations (Eads, Price, & Levine, 2015; Metcalfe-Smith et al., 1998; Ostrovsky & Popov, 2011; Strayer et al., 2004), to our knowledge the validity of these expectations has yet to be widely tested.

A review of literature from the past 50 years using Web of Science, searching for the terms 'freshwater mussel' AND either 'fishway', 'fish ladder', 'passage', or 'recolonisation' (and 'recolonisation') returned a total of 22 results. Thirteen of these papers were eliminated from consideration after reviewing the titles and abstracts and briefly searching for key words. Of the remaining nine, some dealt with mussel recovery associated with fish recolonisation (Metcalfe-Smith et al., 1998; Raitzel & Hartenstine, 2006; Riccardi et al., 2016); however, in these cases there was either no fishway, or methods other than a fishway were used concurrently (e.g. restocking via transplantation, 'trucking' or elevators). It appears that there is only one published study presenting evidence for mussel recolonisation after fishway installation (Smith, 1985), although this study did not include data on abundances so the extent of the recovery was not clear.

An opportunity exists in rivers of southwestern Australia (SWA) to test the prediction that fishways facilitate mussel recolonisation. Despite low levels of aquatic species richness, rivers in this region boast a freshwater fauna with ancient lineages and exceptional endemism (Davies & Stewart, 2013). While freshwater systems in many parts of the world contain multispecies mussel assemblages, SWA has only one species, the endemic Carter's freshwater mussel (*Westralunio carteri*, Iredale 1934) (Walker, Jones, & Klunzinger, 2014). Research on this species was almost entirely neglected until recent work on its ecology, life history and conservation status (Klunzinger, 2012). It is a host generalist, and glochidiosis has been observed on seven of the region's 11 native fish, as well as four exotic species, but successful metamorphosis of glochidia has not been confirmed for most species, and host infestation rates vary among rivers and habitat types (Klunzinger, Beatty, Morgan, Thomson, & Lymbery, 2012b). Although *W. carteri* is widespread and sometimes found in densely populated beds, it is experiencing a decline in distribution and abundance caused by stressors such as secondary salinity, dewatering, and instream barriers (Klunzinger, Beatty, Morgan, Pinder, & Lymbery, 2015). The extent of occurrence has decreased by 49% in less than three generations leading to the species being listed as Vulnerable (VU A2c) by the International Union for Conservation of Nature (Klunzinger & Walker, 2014; Klunzinger et al., 2015). Given that mussels are known to be integral ecological components in freshwater systems globally (Aldridge, Fayle, & Jackson, 2007; Atkinson & Vaughn, 2015; Vaughn & Hakenkamp, 2001), the importance of conserving *W. carteri* in this region is high.

An abundant but disjunct population of *W. carteri* was discovered in Goodga River during a mussel survey on the south coast of SWA in 2012 (Klunzinger et al., 2012a). Although the population was relatively abundant (1–15 mussels/m<sup>2</sup>), no mussels were detected upstream of a low-head hydrographic weir (~1.5 m). The weir was installed in 1964, resulting in the exclusion of all fish species from the upstream reach (Morgan, 2003; Morgan & Beatty, 2006), and it appears that without fish hosts, *W. carteri* was also extirpated from that part of the river. In 2003, a vertical slot fishway was installed at the weir to facilitate upstream spawning migrations of the 'Critically Endangered' spotted trout-minnow (*Galaxias truttaceus* Whitley 1944) (Morgan & Beatty, 2006; Morgan et al., 2016). The fishway was an immediate success, allowing *G. truttaceus* and common jollytail (*Galaxias maculatus*) to expand immediately into upstream habitats (peaking at densities of ~2.70 and 2.75 individuals/m<sup>2</sup> for *G. truttaceus* and *G. maculatus*, respectively, during the 2-year monitoring period) (Morgan & Beatty, 2006), and the population has subsequently grown with increases in both the abundance and maximum sizes of fish (Morgan et al., 2016). Other species of fish are also now present in the upstream reach, including confirmed hosts, the western pygmy perch (*Nannoperca vittata*) and the non-native eastern



*Gambusia holbrooki*) (PGC unpublished data). Given that the upstream and downstream reaches provide similar habitat for mussels, and *W. carteri* is a host-generalist (Klunzinger et al., 2012b), with a number of confirmed and likely hosts in the river, it seems that mussels should have recolonised above the weir together with fish. However, they were not detected upstream during the 2012 mussel survey, 9 years after fishway installation (Klunzinger et al., 2012a).

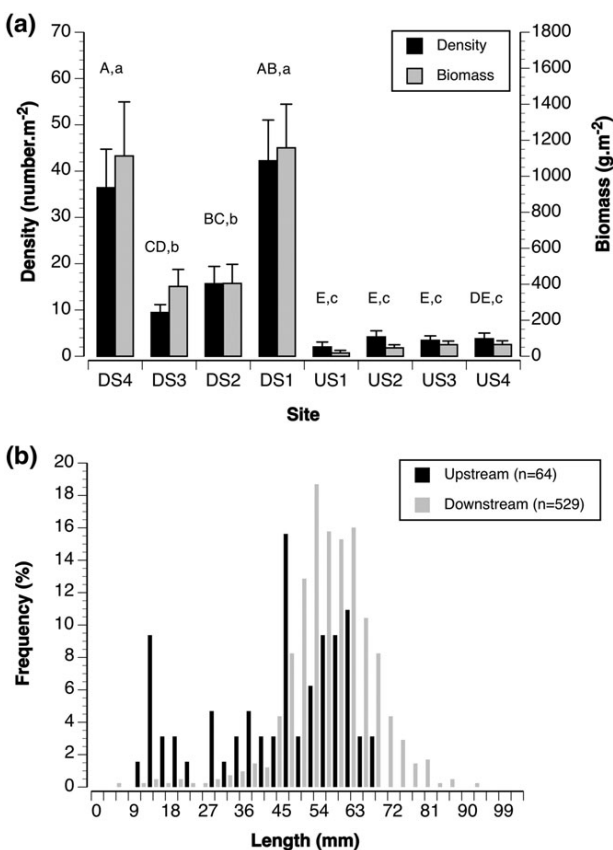
The objectives of this study were:

1. To determine whether mussels had recolonised upstream of the weir after the fishway had been in place for 14 years.
2. In the event that mussels were found upstream, to test the hypothesis that the distribution of size classes would differ between the upstream and downstream reaches, with the upstream having a larger proportion of small size classes, while lacking large mussels.

## Methods

This study was undertaken in Goodga River; located in a small coastal catchment (c. 16 km<sup>2</sup>) on the far south coast of Western Australia (34°56'50.23"S, 118° 4'45.95"E). Sampling for *W. carteri* was undertaken in March 2017 in eight river pools: four sites downstream (DS1–4) and four sites upstream (US1–4) of the fishway. All pools were located within the main channel of the river except DS3, which was disconnected by a narrow sand bank. Before quantitative sampling, the presence of mussels was confirmed at all sites. Quantitative sampling was undertaken using 0.25 m<sup>2</sup> quadrats haphazardly placed within c. 2.5 m of the river bank, where shallow depths allowed tactile searches for mussels (<700 mm water depth). The number of quadrats varied among sites ( $n = 10–27$ ) depending on habitat extent and complexity. In total, samples were collected from 86 and 61 quadrats in downstream and upstream sites, respectively. The substrate within each quadrat was systematically hand-searched for mussels to a depth of about 10 cm for at least 5 min. Hand-searching continued until no additional mussels were collected for 1 min (typical search time c. 10 min). Mussels were counted and the longest shell length (SL) was measured to the nearest 0.1 mm using digital Vernier-calipers before returning the mussel to the river at the collection site.

Estimates of biomass were obtained using a length-wet weight relationship ( $y = 0.0002x^{2.9282}$ ;  $R^2 = 0.9844$ ) for mussels collected from Goodga and nearby rivers during concurrent sampling. The equation was determined from a sample of 163 mussels, which were wiped dry before measuring SL to the nearest 0.1 mm and weighing to the nearest 0.1 g. The differences in mussel density and biomass among pools were tested in *R* using the Kruskal–Wallis test, followed by post-hoc assessment with Dunn's test using the Benjamini–Hochberg correction for false detection rate, in the PMCMR package (Pohlert, 2014; R Core Team, 2017). Differences in size structure of the population downstream and upstream of the fishway were assessed in *R* using a Kolmogorov–Smirnov two-sample test (R Core Team, 2017) ( $\alpha = 0.05$ ). Mussels were classed as small (<45 mm SL), medium (45–65 mm SL), or large (>65 mm SL)



**Figure 1.** (a) Density and biomass of *Westralunio carteri* collected from four pools upstream (US) and downstream (DS) of the fishway on the Goodga River in Western Australia. Significant differences among columns are denoted by upper and lower case letters for density and biomass respectively. (b) Length–frequency (SL) distribution for *W. carteri* collected at sites upstream and downstream of the fishway

for the purpose of discussion.

## Results

Mussels were present in all sites sampled both below and above the weir. The density of mussels in DS pools ranged from 9.4–42.2 mussels/m<sup>2</sup>, while density in US pools ranged from 2.0–4.2 mussels/m<sup>2</sup>. Mussel biomass in DS pools ranged from 387.8–1157.8 g/m<sup>2</sup> compared with 17.8–64.9 g/m<sup>2</sup> in US pools. The mean density ( $\chi^2 = 68.58$ ,  $df = 7$ ,  $P << 0.0001$ ) and mean biomass ( $\chi^2 = 79.64$ ,  $df = 7$ ,  $P << 0.0001$ ) of mussels varied significantly among pools (Figure 1a). Pairwise comparison of density estimates among pools showed that all sites located downstream of the fishway had significantly greater densities compared with those sites located upstream, with the exception of DS3 and US4 ( $P = 0.052$ ; Figure 1a). Similarly, pairwise comparison showed that biomass estimates were significantly greater at sites located downstream of the fishway (Figure 1a).

The size structure within the population varied between the downstream and upstream reaches ( $D = 0.4723$ ,  $P << 0.0001$ ; Figure 1b). The largest mussel collected from US pools was 64.6 mm SL. In DS pools, large mussels (>65 mm SL) were common, with the largest mussel collected from the mainstem being 78.8 mm SL. The isolated pool, DS3, was notable in that it contained mostly large mussels, including the 10 longest collected in this study (79.2–91.2 mm SL). Minimum SLs were consistent with young-of-the-year recruits both upstream (5.8 mm) and downstream (8 mm). The downstream population was dominated by sizes between ~48 and 66 mm SL, while the dominant group upstream was ~51–60 mm SL, with an additional strong cohort in the 42–45 mm SL range. Small mussels (<45 mm SL) made a greater contribution to the size structure upstream when compared with downstream, and the density of this size class was similar upstream and downstream (2.3 and 2.2 mussels/m<sup>2</sup> respectively, Figure 1b).

## Discussion

This paper provides evidence that the ecological benefits of fishways extend to invertebrate fauna, and adds support to various studies highlighting the detrimental effect of instream barriers on mussel populations (Araujo & Álvarez-Cobelas, 2016; Brainwood, Burgin, & Byrne, 2008; Watters, 1996). Previously thought to be restricted to habitats downstream of the Goodga weir (Klunzinger et al., 2012a), the study recorded *W. carteri* at all sites sampled upstream of this barrier. Combined with density and biomass estimates, size structure comparisons indicate that there has been strong upstream recruitment following the installation of the fishway in 2003, and suggest that the population will continue to recover above the weir. The low densities and biomass found upstream reflect the lack of recruitment post-weir and pre-fishway, as well as the slow rate of recovery expected for mussels, which grow slowly and are late to mature (Strayer et al., 2004). Lack of detection in 2012 was probably due to lower abundances at the time of sampling, as well as the cryptic nature of young mussels, which includes behavioural differences (e.g. rapid and more frequent burial), distinct microhabitat preferences, and very small body size (Hastie et al., 2000).

The demographics of the downstream population indicate long-term persistence of *W. carteri* at Goodga, and in the absence of a weir, population structure would not be expected to differ so dramatically between the upstream and downstream reaches. Biological surveys of the river have been scarce until the last decade, so there is an absence of pre-fishway data with which to compare the present findings. The discovery of the downstream population by Klunzinger et al., (2012a) appears to have been the first mussel survey at Goodga. Before that study there were only two surveys of the broader invertebrate community, the first of which did not find mussels despite sampling in the

downstream reach, albeit at just one location (Shiel, 1993), while the second was limited to upstream habitat, and did not detect mussels during four sampling events from 1994-1996 (Halse, Scanlon, & Cocking, 2002). The lack of large mussels in the upstream reach during the present survey suggests that there was little or no recruitment before fishway installation 14 years ago. Age-at-length measurements for other populations of this species (Klunzinger et al., 2014) show that SLs greater than 65 mm are not reached until 15+ years, at which point growth reaches an asymptote, with maximum lengths of 75-80+ mm not reached for 25–50+ years. Mussels ranging from 65-79 mm SL were common downstream of the weir in the mainstem of the river, but the largest individual found upstream was only 64.6 mm SL. Although mussel growth rates vary with environmental factors such as temperature and food availability (Hastie, Young, & Boon, 2000), it is unlikely that differing growth rates could account for the smaller maximum sizes in the upstream reach given the proximity of all of the sites (within 2.5 km river length), and similarities in habitat and physio-chemical conditions. The similar density of small mussels (<45 mm SL) both upstream and downstream indicates that recruitment is now comparable in both habitats. If recruitment had been unchanged over the past 53 years (the time since the weir was installed) then the density of larger size classes would not be expected to differ so dramatically. The largest mussels found upstream of the weir are almost certainly far younger than those found downstream.

The results also suggest that the density of *W. carteri* has recently increased downstream of the weir. The downstream densities reported in this study generally exceed the 1–15 mussels/m<sup>2</sup> previously reported for the same reach of river (Klunzinger et al., 2012a). Three of the four DS pools sampled here exceeded 15 mussels/m<sup>2</sup> (maximum density in DS1 of 42.2 mussels/m<sup>2</sup>). Although the change in densities could reflect possible differences in sampling effort between the two studies, there are reasons to suspect that the increase in abundance is real. First, the *W. carteri* population may have mirrored the growth of the *G. truttaceus* population, which has increased in overall numbers and maximum lengths of individuals (Morgan et al., 2016), potentially providing more host fish (sensu Arvidsson et al., 2012). Second, the *G. truttaceus* population boom would also presumably translate to a greater abundance of juvenile fish, which undertake a spring mass migration (Close et al., 2014; Morgan et al., 2016) coinciding with the period of glochidial release by *W. carteri* (Klunzinger et al., 2012b). Third, the Goodga mussel population was only discovered in 2012 (Klunzinger et al., 2012a); while there have been few biological surveys of the river, mussels were not detected during an invertebrate survey in the downstream reach by Shiel (1993), despite extensively sampling instream habitats including various benthic substrates, albeit at only one location. The apparent high densities of mussels at present compared with those previous reports suggests a recent population boom throughout the river.

Dams and weirs can greatly alter river ecosystems, having multiple adverse effects on freshwater organisms (Dudgeon et al., 2006; Lydeard et al., 2004; Rolls et al., 2013), and the removal of barriers is often a necessary part of stream restoration (Gangloff, 2013). In some cases, however, small impoundments may provide unexpected ecological benefits such as filtering elevated nutrient loads, capturing fine sediments, and stabilising streambeds (Gangloff, 2013). In drying climates, the pools that impoundments create may also be a valuable refuge for freshwater fauna during extended drought (Beatty et al., 2017). In such cases, it may be preferable to keep the barrier in place and seek an alternative solution. The present study provides an example of connectivity for mussels being successfully restored without destroying the barrier that fragmented the population. The applicability of this approach to other situations will depend on factors such as the host-specificity of the mussel and mobility of the host. For example, *W. carteri* is a host-generalist, likely to use highly mobile galaxiids (Klunzinger et al., 2012b). These fish are strong swimmers and are known to use the Goodga fishway in large numbers (Morgan & Beatty, 2006). Other fish may avoid high-flow environments or be less able to traverse them, and fishways would be unsuitable for the recovery of mussels that are limited to such hosts. The relationships between mussels and their hosts remain unstudied or incompletely explored in many cases. Interactions between native fish decline and the expansion of invasive species, and the effect of habitat on host compatibility in altered rivers will make these relationships increasingly complicated to unravel. These knowledge gaps constrain the identification of an appropriate approach to restore connectivity. Removal of barriers should not be pursued dogmatically, but rather considered within the regional context, with consideration given to potential positive and negative outcomes (Gangloff, 2013; Beatty et al., 2017). Where removal is not seen as a suitable action, fishways may provide an alternative approach.

This study provides one of the first formally documented examples of invertebrate fauna benefiting from the installation of a fishway. It is expected that the expansion of the *W. carteri* population within Goodga will benefit the ecosystem, as mussels are important ecological components of freshwater ecosystems due to their engineering of physiochemical habitat conditions, and for the often significant contribution they make to benthic biomass. That it has taken this species 14 years since the installation of the fishway to reach detectable densities in the upper river highlights the long-lasting impact that barriers can have on freshwater fauna. It is promising, however, that recruitment in this area appears to be consistent with reinstating fish passage using a fishway, suggesting that the population will grow and that mussel-derived ecosystem services will increase in time.

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## Chapter 4:

# Freshwater tributaries provide refuge and recolonisation opportunities for mussels following salinity reversal



**Cover picture:** Kent River (top), Styx Creek (bottom left), and Nile Creek (bottom right)

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## Abstract

Reversing the effects of secondary salinisation, and its impacts on freshwater biodiversity, is a growing global challenge, and particularly prevalent in Mediterranean-climate regions. Remnant freshwater tributaries in salinised landscapes provide significant biodiversity values, including discrete areas of refuge, dilution of salinised reaches, and potential source populations for recolonisation. The importance of these areas for freshwater fauna is widely accepted but rarely evaluated in the field. This study explored how the spatial distribution of southwestern Australia's only freshwater mussel species, *Westralunio carteri*, has responded to the ongoing salinity trend in the Kent River catchment. Our results showed that salinity in the river has begun to reverse following improved catchment management, and detected the first evidence of an associated recovery of the freshwater mussel population. Mussels in the mainstem were limited to sites around and downstream of a permanently flowing freshwater tributary, suggesting that dilution from this source provides a refuge in the lower reach. At two of those sites, all individuals were <15 years of age, indicative of recolonisation coinciding with salinity reversal around the turn of the century. Interestingly, mussels clearly persisted in other parts of the lower reach throughout the peak salinity period, when salinities regularly exceeded laboratory derived toxicity thresholds for the species. Mussels were not found in the majority of the mainstem or in highly acidic parts of the freshwater tributaries. The presence of old shells at those sites shows that the species was once widespread, and that the current distribution probably reflects a contraction due to historical salinisation as well as acidification. Overall, our results show that the *W. carteri* population in the catchment has taken a first step towards recovery from salinisation, and highlights the importance of freshwater tributaries in providing both refuge from disturbance and a source of new recruits.

## Introduction

Secondary salinisation is a growing threat to freshwater biodiversity globally, significantly modifying ecosystem structure and function, and it is not known whether its impacts can be reversed (Cañedo-Argüelles et al., 2013; Herbert et al., 2015; Cañedo-Argüelles, Kefford, & Schäfer, 2019). Mediterranean-climate (med-) regions are heavily impacted by secondary salinity, and the issue is exacerbated by coexisting stressors, such as acidification, water abstraction, and climate change (Degens et al., 2012; Robson et al., 2013; Estevez et al., 2018). In the med-region of southwestern Australia, widespread clearing of native vegetation has caused approximately 56% of the region's large rivers to turn brackish or saline (Mayer, Ruprecht, & Bari., 2005; Beatty et al., 2011), and much of the regions highly endemic freshwater fauna is now restricted to remnant freshwater tributaries closer to the coast (Beatty et al., 2011; Pettit et al., 2015). In recent years, legislated control on land clearing, and revegetation of the agricultural inland area has helped to slow secondary salinisation, and salinity has even begun to reverse in some rivers (Mayer, Ruprecht, & Bari., 2005). There is the potential for salt-sensitive fauna that have persisted within freshwater refuges to recolonise these formerly salinised areas, but there is as yet no evidence for this having occurred (Robson et al., 2013).

Freshwater mussels (*Bivalvia*, Unionida; hereafter 'mussels') are one of the worlds most threatened faunal groups (Hastie et al., 2000; Strayer et al., 2004; Lopes-Lima et al., 2017), and their ongoing decline has severely impacted ecosystem function (Vaughn, Atkinson, & Julian, 2015; Vaughn, 2018). Mussels are highly vulnerable to salt pollution (e.g. Todd & Kaltenecker, 2012; Patnode et al., 2015), particularly at the juvenile and larval (glochidial) stages (Blakeslee et al., 2013; Prosser et al., 2017). As an obligate parasite, glochidia are further impacted because elevated salinities can reduce attachment success to host-fish (Beggel & Geist, 2015), and many freshwater fish may be excluded from salinised reaches (Beatty et al., 2011). While the salt-sensitivity of early life stages would be expected to impact new recruits in extant populations, field studies rarely assess recruitment (however see Johnson, Krstolic, & Ostby, 2014), and most studies report only mussel presence-absence (e.g. Klunzinger et al., 2015), or species abundance (e.g. Zipper et al., 2016). Demographic studies may be particularly important because many mussel species are long lived (sometimes >100 years), meaning that populations can persist for years without recruitment, resulting in an extinction debt (Haag, 2012; Ferreira-Rodriguez et al., 2019). In combination, these factors make mussel populations unlikely to recover quickly from disturbances (Strayer et al., 2004), and the potential for natural recolonisation after extirpation is still mostly unstudied (for examples of recovery from other disturbances see: Riccardi et al., 2016; Gillis et al., 2017;

Benson et al., 2018). To our knowledge, there have been no studies that have investigated mussel population recovery following salinity reversal, and only one has addressed the effect of salinity on mussel distribution in salinised med-climate rivers (Klunzinger et al., 2015).

Carter's freshwater mussel (*Westralunio carteri*, Iredale 1934; IUCN Red List: Vulnerable (VU A2c)) is the only mussel species in southwestern Australia (Walker, Jones & Klunzinger, 2014). While *W. carteri* was once widespread and sometimes highly abundant, its extent of occurrence has declined by 49% in the last three generations largely due to secondary salinisation (Klunzinger et al., 2015). The species is almost never found in rivers where mean annual salinity exceeds 1.6 g/L (Klunzinger et al., 2015). The first acute salinity tolerance trials for the species found that it had an LD<sub>50</sub> of 1.6–3.0 g/L, and a LD<sub>95</sub> of 3.6–4.25 g/L, and no individuals survived for longer than eight days at >5 g/L (Klunzinger et al., 2015). Subsequent trials; however, found that *W. carteri* may actually be more robust (LD<sub>50</sub> = 5.87–5.96 g/L; LD<sub>95</sub> = 6.01–6.12 g/L), and that survival was enhanced when using (1) gradual rather than acute salinity increases, (2) more natural feeding conditions, and (3) individuals collected from higher salinity habitats (Ma, 2018). These findings suggest that *W. carteri* has the capacity to respond to increasing salinity through both physiological acclimatisation and genetic adaptation (Ma, 2018). In addition, the natural environment may provide spatial and temporal refuges that allow species to persist in otherwise unsuitable habitats (Robson et al., 2013). Conversely, natural environments may present additional stressors that exclude species from apparently suitable habitats (Beermann et al., 2018). While acute salinity trials are valuable for determining tolerances and showing short-term responses, the effect of life-long (>50 years) exposure at sublethal levels on population structure in natural settings is not known, and requires validation in the field.

An opportunity existed within the Kent River catchment in southwestern Australia to assess mussel distribution and population structure at a range of salinities in a highly salt-impacted landscape. Rapidly rising salinity in the Kent River was combated in the 1970s and 80s through clearing control and revegetation in the upper catchment. By 2002, these strategies had slowed the rising rate of salinity and it was expected that it would eventually begin to decline towards pre-disturbance levels (Mayer, Ruprecht, & Bari., 2005; De Silva et al., 2007). It is likely that *W. carteri* was once widespread through the river, but there are no pre-salinisation distribution data available. Current salinity levels are among the highest where the species is known to occur, and mussels have only been detected in two fresh tributaries, as well as in the lower reach of the mainstem below the lower tributary (Klunzinger et al., 2012a; 2015).

The objective of the current study was to assess the ongoing salinity trend in the Kent River, and to explore how mussel spatial distribution and abundance has responded to that trend. Our hypotheses were that:

1. Long-term salinity data would show that salinity has begun to decline since 2002.
2. This decline has facilitated increased mussel recruitment and recolonisation in the mainstem of the river.
3. Mussels would be more abundant in freshwater tributaries than in brackish sites in the mainstem, while being entirely absent in higher salinity areas (>3-4.25 g/L).
4. Although we expected mussels to be absent from high salinity sites, we predicted that we would find evidence (i.e. old shells) of previous occupation at those sites.

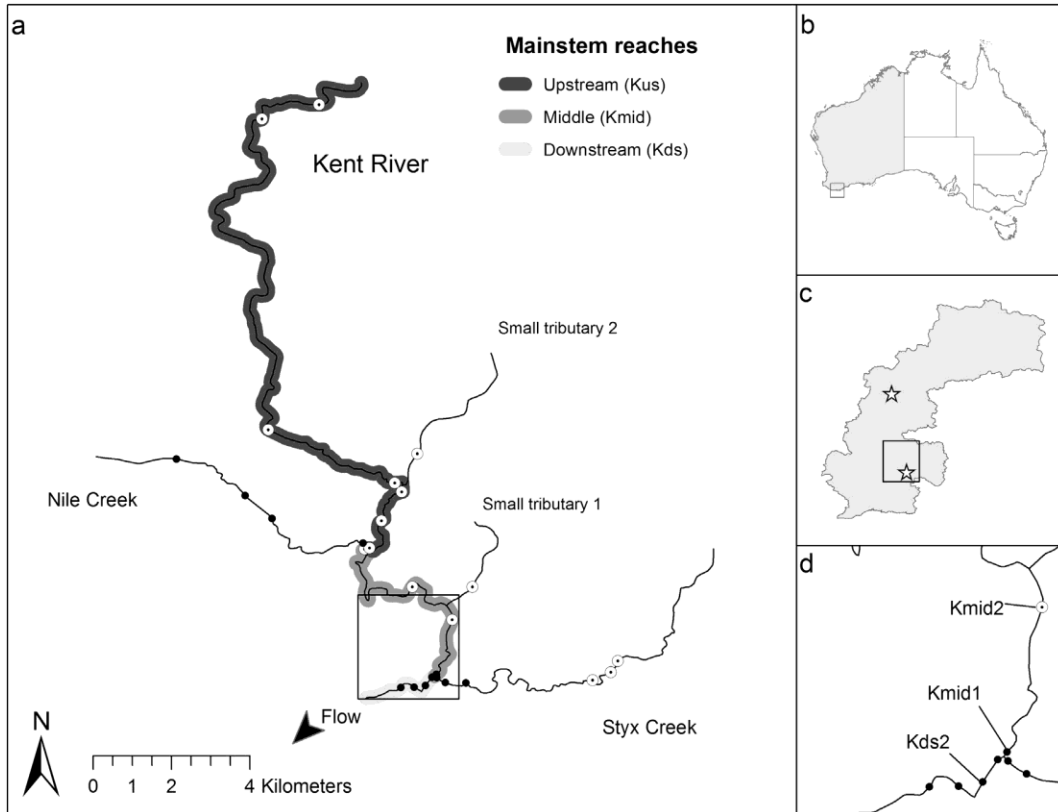
## Methods

### **Study area**

The effect of salinity on *W. carteri* distribution was assessed in the lower catchment of the Kent River (between 34°45'05"S and 34°53'20"S; Figure 1a) on the south coast of southwestern Australia. The Kent River originates 80 km inland and drains a total area of c. 2500 km<sup>2</sup>. Two hydrographic stations continuously monitor conductivity and discharge on the mainstem of the River; one north of the study area, ~45 km inland at Rocky Glen, and the other within the study area, ~15 km inland, below the confluences of two freshwater tributaries (Nile Creek and Styx Creek) (Figure 1c). The Kent River is 'moderately saline' to 'saline' at the upper station (mean annual salinity ~4.17 g/L) and 'marginal' to 'brackish' at the lower station for most of the year (mean annual salinity ~1.65 g/L) (Mayer, Ruprecht, & Bari., 2005). At the onset of winter rainfall, flushing of the salinised upper catchment results in a pulse of saline water down the river, and mean monthly salinity reaches as high as ~8.88 g/L and ~3.05 g/L at the upper and lower stations respectively (Mayer, Ruprecht, & Bari., 2005). Nile Creek is located within an almost entirely uncleared catchment, with pristine natural vegetation. Styx Creek enters the Kent River ~10 km downstream of Nile Creek, and drains a mostly uncleared catchment with farmland bordering the lower reach.

Twenty-seven sites in the lower catchment that contained physical habitat likely to support *W. carteri* were sampled from November to January in 2016-17 and 2017-18 (Figure 1a). We defined sites as being 'suitable' for *W. carteri* if the salinity level in a single surface water grab sample was below the acute tolerances for the species derived from the Collie River (LC50 = 3 g/L; LC95 = 4.25 g/L; Klunzinger et al.,

2015). The uppermost site in the mainstem (Kus7) was 'unsuitable' as it exceeded these threshold values (>5 g/L), while all sites downstream of that point were <2.9 g/L. Four sites were sampled on Nile Creek (N1-N4), six on Styx Creek (S1-S6), and two small perennial creeks in the upstream reach (UStrib1 and UStrib2) were sampled at one location each. The Kent River was sampled at four locations downstream of Styx Creek (Kds1-Kds4), at four locations between Styx Creek and Nile Creek (Kmid1-4), and at seven locations upstream of Nile Creek (Kus1-Kus7). Three sites were surveyed at the Nile-Kent and Styx-Kent confluences (within 25-50 m of the confluence upstream, downstream, and in the tributary). Each sampling site consisted of a river segment 50 m long.



**Figure 1.** (a) Site locations in the Kent River catchment showing *Westralunio carteri* presence and absence (full and open circles respectively). The mainstem of the river is in bold and is divided into three reaches (see legend). Inset maps show the locations of (b) the catchment in southwestern Australia, (c) the study area within the catchment including the locations of the upper and lower hydrographic gauging stations, and (d) the three sites around the Styx confluence where continuous salinity data were collected. The location of panel (d) at the Styx confluence is outlined on panel (a). Note: site Kds2 was located at the lower gauging station



### ***Physiochemical parameters***

This study incorporated salinity records from field measurements, hydrographic gauging stations, government reports, and peer reviewed literature. These sources variably reported salinity as conductivity compensated to 25°C ( $\mu\text{S}/\text{cm}$ ), total dissolved solid (TDS mg/L), and salinity (g/L). To enable comparison, all values were converted to salinity (g/L) as follows: conductivity ( $\mu\text{S}/\text{cm}$ ) measurements were converted using the formula  $S = 0.4665x^{1.0878}$  (Williams, 1986); TDS mg/L measurements (Mayer, Ruprecht, & Bari., 2005; De Silva et al., 2007) were converted to conductivity<sup>25</sup> by dividing by 0.5333 (the specific TDS-conductivity relationship for the Kent River, obtained from those reports) before being converted to salinity using Williams (1986).

In situ measurements of pH, oxidation reduction potential (ORP), dissolved oxygen (%), turbidity (FNU), temperature (°C), and salinity (g/L) for each site were recorded using a Hannah multi-parameter probe between the 3rd and 9th January 2018 between the hours of 10am and 2pm in order to minimise temporal variability. Long-term salinity data spanning the period January 1979 to September 2018 were obtained from the lower hydrographic station below the confluence with Styx Creek. The reliability of hydrographic station salinity data was assessed using quality codes provided with the data. Data quality for 1991 was listed as “not reviewed/quality unknown” and so that year was excluded from analysis. Mean salinity measurements for each day were used to calculate an annual mean for each complete year of data, as well as determine how often stream salinity exceeded acute tolerance thresholds for *W. carteri* (LC<sub>50</sub>, LC<sub>95</sub>, and 5 g/L as determined by Klunzinger et al., 2015) and how long these events lasted. An event was defined as a series of consecutive days above a threshold level. Odyssey conductivity loggers were deployed at two sites in the Kent River above the confluence with Styx Creek (Kmid1 and Kmid2) (Figure 1d). Combined with the hydrographic station at Kds2, this provided high spatial resolution salinity data for that section of the river. Odyssey loggers were deployed on 4<sup>th</sup> December 2017 and collected on 1<sup>st</sup> October 2018. Hydrographic data for that same period were obtained from the upper and lower hydrographic gauging stations to investigate the relationship between salinity and flow.

### ***Mussel sampling***

Each site was sampled for mussels by one worker for 1 hour using tactile searches combined with visual searching where possible (i.e. visibility c. 1-2 m). An effort was made to search habitat likely to contain juvenile mussels (e.g. flow refuges and deeper sediment); however, complete sediment excavations would be needed to fully assess abundance of young of the year and smaller individuals. As such, the methods employed are considered to be semi-quantitative. All live mussels were measured for longest

shell length (SL) with digital Vernier calipers to the nearest 0.1 mm and returned to the water. The abundance of live mussels at each site was recorded as the number of individuals collected per hour and reported as catch per unit effort (CPU).

### ***Data analysis***

Salinity data from the lower hydrographic station were analysed in River Analysis Package (RAP) to assess mean annual salinity in the Kent River over the period 1979-2017, as well as the frequency and duration of high salinity events in 15 year periods pre- and post-2002 (i.e. the endpoint of the previous assessment of salinity in the river; Mayer, Ruprecht, & Bari., 2005). Mann-Whitney U tests were used to compare the mean frequency and duration of recent annual salinity events (2003-2017) to historical levels (1987-2002 excluding 1991). Salinity data for 2018 from the lower hydrographic station were combined with Odyssey logger data to describe salinity dynamics around the Styx Creek confluence.

The effect of all measured water quality parameters on mussel presence was tested using a generalised linear model, with a binomial distribution and a logit link function. Preliminary models indicated that temperature and turbidity explained very little of the variance in mussel presence, so they were removed from the final model, which included salinity, pH, dissolved oxygen and oxidation reduction potential. As our goal was to determine the relative importance of environmental factors, and not to develop a predictive model (Bumham & Anderson, 2002), we also used a multimodel inference approach to determine variable importance. The global model (including all four predictor variables) was used to generate a set of all possible models using the *R* package MuMIn (Barton, 2013). Models were ranked by the Akaike Information Criterion, and the importance of each variable was determined by the number of times it occurred within the top-ranked set (those within 4 AIC values of the best model).

Mean CPU, with bootstrap 95% confidence intervals, were calculated for sites where mussels were found. A Kruskal-Wallis test was used to compare mean CPU of mussels in sites on the Kent, Styx and Nile where mussels were found. Length-frequency data for Kent River sites were used to assess recruitment in the mainstem of the river using the proportion of individuals <54 mm SL (approximately 10 years of age (Klunzinger et al., 2014)) at each site as an indicator of recent recruitment. Differences in recruitment among sites were compared using a Kruskal-Wallis test. Post-hoc comparison was performed with pairwise Wilcoxon tests using Holm's adjustment. All statistical analyses were performed in *R* (R Core Team, 2018).

## Results

### ***Long term salinity trend in the mainstem***

Salinity in the Kent River peaked near the turn of the century and has now begun to decline (Figure 2a). In the 15 year period since 2002 the number of events exceeding the LC<sub>50</sub>, LC<sub>95</sub>, and 5 g/L thresholds for *W. carteri* has decreased significantly compared to the previous 15 year period (45.8%, 70.0%, and 65.0% decreases respectively;  $P < 0.05$  for all, Figure 2b). There was also a significant decrease in the number of days per year above those thresholds (70.1%, 88.1%, and 92.5% decreases respectively;  $P < 0.05$ , Fig 2c). The longest event exceeding the LC<sub>50</sub> threshold lasted for 40 or more consecutive days in 1987, 1998 and 2002 (Figure 2d). In that last event, the LC<sub>95</sub> and 5 g/L levels were also exceeded for 23 and 13 consecutive days respectively (Figure 2e and f). No LC<sub>95</sub> or 5 g/L event has lasted longer than 5 and 2 days respectively since 2002. In 2010 and 2013 there were no events exceeding any threshold for the first time since 1982.

### ***2017/18 salinity data in the mainstem***

Mean daily salinity at Kds2 was below the LC<sub>50</sub> level (3 g/L) for all but two days of the sampling period peaking at ~3.92 g/L (Figure 3a). Mean daily salinity at Kmid1 showed a similar temporal trend to Kds2, but was generally 0.5-1 g/L higher despite being <500 m upstream, peaking at 4.24 g/L (Figure 3a). Mean daily salinity at Kmid2 was distinctly higher than at Kmid1 despite being <2 km upstream and there being no obvious freshwater inputs between them, and exceeded the LC<sub>50</sub> and LC<sub>95</sub> thresholds for the majority of the sampling period, peaking at 5.2 g/L.

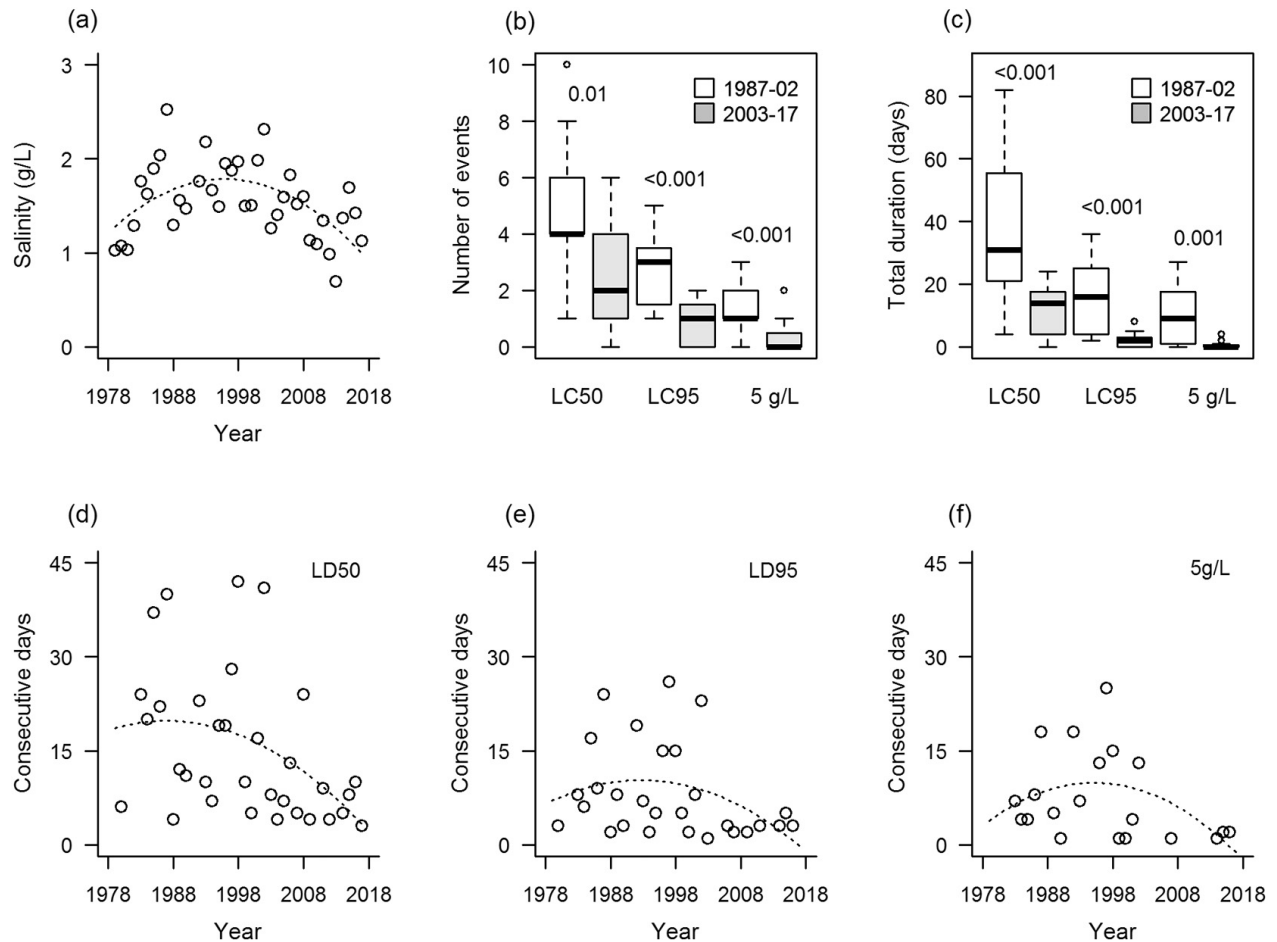
Peak salinity at all mainstem sites coincided with increased flow from the saline upper catchment at the onset of winter (Figure 3b). Although flow stopped at the upper gauging station, in the lower reach it was maintained throughout the study period by freshwater input from Styx Creek (Figure 3c). It was noted during field work that Nile Creek stopped flowing near the confluence by January (pers. obs. JB).

### ***Mussel distribution and abundance in the mainstem and tributaries***

No mussels were found at sites with salinity >1.48 g/L or with pH <4.5 (highly acidic; Degens et al. (2012)), and both factors had a significant effect on mussel distribution (GLM,  $P < 0.001$ ; Table 1).

Mussels were common at all sites in Nile Creek (82-465 CPU) as well as in the three lower sites in Styx Creek (115-194 CPU; Table 2). All other tributary sites lacked mussels despite being fresh (salinity <0.69 g/L); those sites were highly acidic and >20 degraded shells and various shell fragments were found in the sediment at S5. Mussels were only detected at five mainstem sites, four of which were downstream

of Styx Creek while the fifth was 50 m upstream of the confluence. Kmid1 and Kds1 had the lowest CPU of all sites (8 and 38 mussels respectively) while Kds3 and Kds4 had among the highest CPU (404 and 505 mussels respectively). Numerous old shells were collected while snorkeling in deep pools (1-4m deep) at the uppermost sites, Kus6 and Kus7, indicating that mussels were historically present at least that far upstream (~20 km and 30 km upstream of Nile and Styx Creeks respectively). There was no significant difference in mean CPU among reaches (Kent, Styx and Nile) using only sites where mussels were detected (Kruskal-Wallis:  $P > 0.05$ ; Table 2).



**Figure 2.** (a) Mean annual stream salinity in the Kent River from 1979 to 2017. (b) Number of salinity events per year that exceeded various toxicity thresholds for *W. carteri* in the 15 year period up to 2002 (white boxplots) compared to the 15 year period after 2002 (grey boxplots), and (c) number of days per year exceeding those same thresholds. Probabilities refer to comparisons of numbers of salinity events or days exceeding threshold levels before and after 2002. (d–f) Longest salinity event each year that exceeded toxicity thresholds. Data were obtained from the Department of Water gauging station in the mainstem of the Kent River below the confluence with Styx Creek. Note, 1991 was excluded from all analysis due to uncertain quality of the data

**Table 1.** Association between water quality variables and *Westralunio carteri* presence ranked by importance (probability of being selected in the Akaike Information Criterion best-fit model), with coefficient estimates and standard errors (SE). Significant predictors ( $P < 0.05$ ) are shown in bold

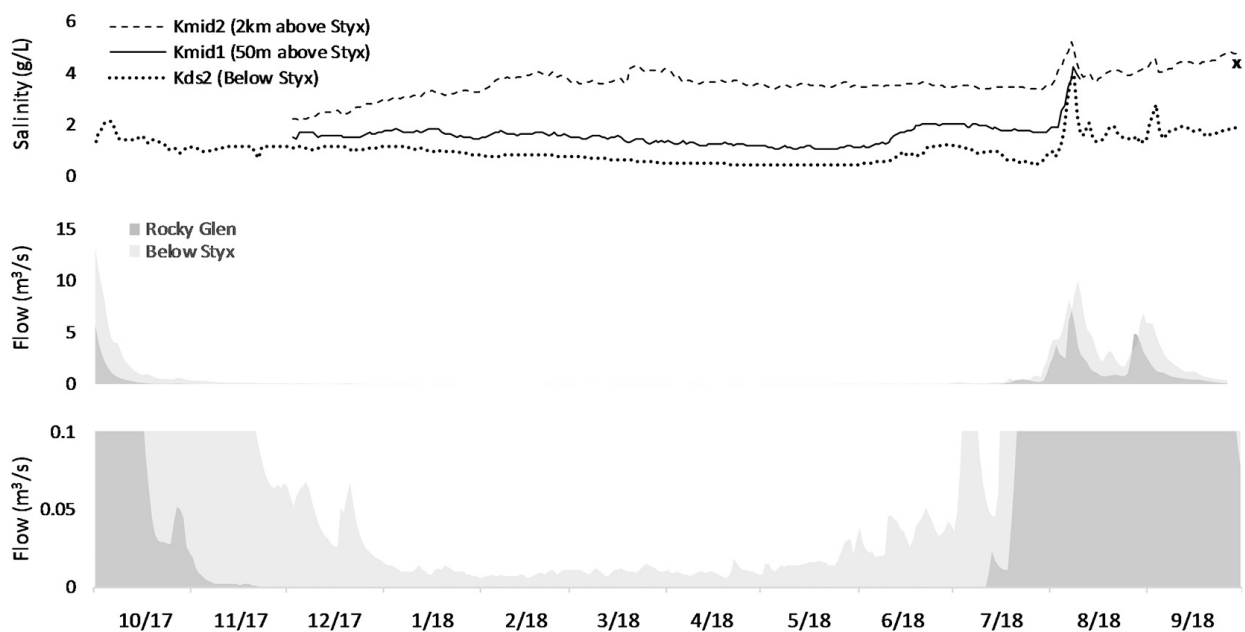
| Variable        | Importance  | Coefficient estimate (SE) | $\chi^2$ (prob)            |
|-----------------|-------------|---------------------------|----------------------------|
| <b>Salinity</b> | <b>1.00</b> | <b>6.10 (2.64)</b>        | <b>24.74 (&lt; 0.0001)</b> |
| <b>pH</b>       | <b>1.00</b> | <b>-2.83 (1.32)</b>       | <b>15.33 (&lt; 0.0001)</b> |
| ORP             | 0.55        | 0.02 (0.02)               | <b>2.23 (0.14)</b>         |
| DO              | 0.55        | 0                         | <b>1.00</b>                |

**Table 2.** Catch per unit effort for *Westralunio carteri* (CPU; 95% Confidence Intervals) and water quality parameters (mean and SD) from sites in the Kent River catchment

|           | Reach                                  | CPU<br>95% CI | pH             | ORP                | DO<br>(%)        | Salinity<br>(g/L) | Turbidity<br>(FNU) | Temp<br>(°C)    |
|-----------|--|---------------|----------------|--------------------|------------------|-------------------|--------------------|-----------------|
| Mainstem  | Upstream of Nile                       | 0             | 7.28<br>(0.63) | 202.43<br>(58.25)  | 93.11<br>(13.71) | 2.64<br>(1.17)    | 4.74<br>(7.88)     | 22.59<br>(3.59) |
|           | Between Nile - Styx<br>excluding Kmid1 | 0             | 7.39<br>(0.56) | 172.02<br>(68.43)  | 88.34<br>(14.41) | 2.57<br>(0.96)    | 4.62<br>(6.63)     | 22.0<br>(3.1)   |
|           | Downstream of Styx<br>including Kmid1  | 43.6 - 393    | 6.93<br>(0.16) | 156.54<br>(29.68)  | 66.52<br>(18.05) | 1.35<br>(0.15)    | 2.7<br>(1.04)      | 22.2<br>(1.0)   |
| Tributary | Small perennial<br>tributaries 1 & 2   | 0             | 4.20<br>(0.39) | 253.45<br>(63.43)  | 67.55<br>(5.59)  | 0.33<br>(0.03)    | 11.65<br>(1.77)    | 18.11<br>(4.08) |
|           | Nile Creek                             | 90 - 377      | 6.79<br>(0.76) | 156.05<br>(52.87)  | 71.85<br>(17.5)  | 0.42<br>(0.07)    | 8.5<br>(3.41)      | 21.9<br>(4.5)   |
|           | Styx Creek: sites 4-6                  | 0             | 4.09<br>(0.19) | 229.63<br>(100.89) | 84.9<br>(17.53)  | 0.61<br>(0.07)    | 15.13<br>(4.32)    | 24.2<br>(3.2)   |
|           | Styx Creek: sites 1-3                  | 115-177       | 6.02<br>(0.59) | 155.33<br>(9.4)    | 50.83<br>(39.54) | 0.44<br>(0.08)    | 16.67<br>(3.98)    | 9.5<br>(1.6)    |

### Mussel recruitment at mainstem sites

Length-frequency distributions show that mussel recruitment has varied among mainstem sites over the past few decades, being historically limited at the three upper sites compared to the two lower-most sites. The proportions of larger individuals (54+ mm SL, equal to 10+ years of age) contributing to populations at Kmid1 (12.5%), Kds1 (21.1%), and Kds2 (25%) were not significantly different but were all significantly less than at Kds3 (69.9%) and Kds4 (80.4%) (pairwise-Wilcoxon test,  $P < 0.05$  for all) (Figure 4). Small *W. carteri* (<54 mm SL, equivalent to <10 years of age) were present at all mainstem sites where mussels were detected, indicating that recent recruitment has occurred. No young-of-the-year were recorded at any of the sites sampled. All mussels found at Kmid1 were <54.8 mm SL, and are all likely to be post-2002 recruits based on SL and examination of external annuli. Similarly, all mussels at Kds1 were <65 mm SL, and therefore likely to be <15 years old (based on Klunzinger et al., 2014; Benson et al. 2018).

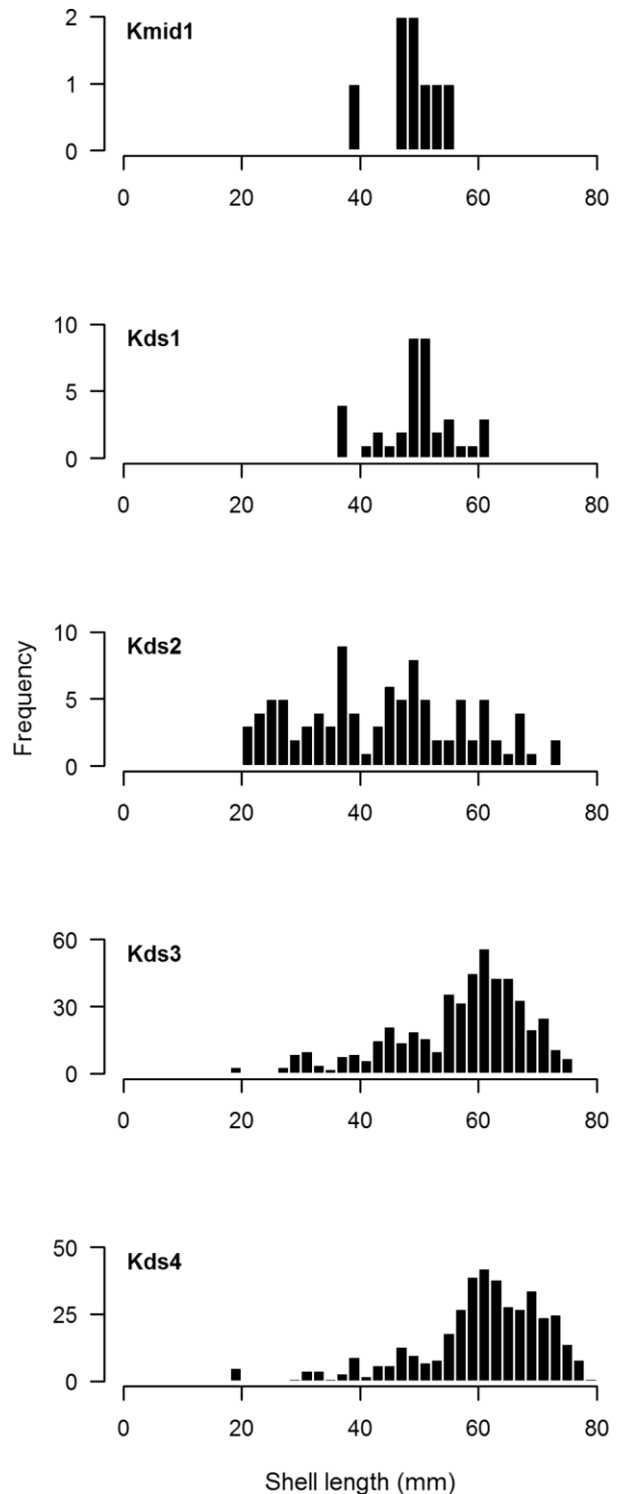


**Figure 3.** The relationship between mean daily flow and salinity in the Kent River. (a) Mean daily salinity at three sites around the confluence with Styx Creek. Note, the logger at Kmid1 stopped working in August, so that series includes a spot measurement (x) taken on the final day during logger retrieval. (b) Mean daily flow at the upper (Rocky Glen) and lower (below Styx Creek) hydrographic stations on the mainstem of the river. (c) The same flow data magnified to show that flow stopped at the upper gauging station from late-2017 to mid-2018, while in the lower reach in was maintained by freshwater input from Styx Creek. (Data were obtained from:

<http://kumina.water.wa.gov.au/waterinformation/telem/stage.cfm>)

## Discussion

Reversing the effects of secondary salinisation, and its impacts on freshwater biodiversity, is a growing global challenge (Cañedo-Argüelles et al., 2013; Herbert et al., 2015; Cañedo-Argüelles, Kefford, & Schäfer, 2019). This study adds to a small number of examples of salinity caused by landscape clearing being successfully reversed through improved catchment management (Mayer, Ruprecht, & Bari., 2005), and we believe it is the first to detect the associated recovery of a mussel population. While *W. carteri* was not found in the majority of the Kent River, the presence of old shells at salinised sites shows that the species was once widespread, and that the current distribution likely reflects a contraction due to historical salinisation. Mussels in the mainstem were limited to sites around and downstream of a permanently flowing tributary, suggesting that dilution from this source provides a freshwater refuge in the lower reach. At two of those sites, all individuals were likely <15 years of age, indicative of recolonisation coinciding with salinity reversal around the turn of the century. Interestingly, mussels clearly persisted in other parts of the lower reach throughout the peak salinity period, and those sites had among the highest densities recorded in this study. Overall, our results show that biodiversity in the catchment has taken a first step towards recovery from salinisation, and highlights the importance of freshwater tributaries in providing both refuge from disturbance and a source of new recruits.



**Figure 4.** Length frequency (SL) distributions for *Westralunio carteri* at five sites on the Kent River, arranged from upstream (top) to downstream (bottom). The Styx confluence is intermediate to Kmid1 and Kds1. Note difference in y-axis values

### *Spatial variability in mussel decline and persistence*

Secondary salinisation has dramatically altered the structure and function of freshwater ecosystems globally (Cañedo-Argüelles et al., 2013; Herbert et al., 2015; Cañedo-Argüelles, Kefford, & Schäfer, 2019), and is the primary driver of *W. carteri* decline in southwestern Australia (Klunzinger et al., 2015). In this study, *W. carteri* was not found in the mainstem >50 m upstream of Styx Creek confluence, and the presence of old shells at our upper-most sites indicates that the species has been lost from at least 30 km of the river. Additionally, until the recent salinity reversal there was no recruitment occurring at sites immediately surrounding Styx Creek confluence, highlighting the precarious position of mussels in the mainstem. Without legislated control on land clearing, and revegetation of the upper catchment, salinity would have continued to rise and *W. carteri* could have been extirpated from the downstream reach as well.

In salinised catchments, freshwater tributaries serve as discrete areas of refuge and also dilute mainstem reaches (Beatty et al., 2011; Robson et al., 2013). This study found that *W. carteri* was abundant in the two largest tributaries of the Kent catchment, often being found within metres of the mainstem. Despite this proximity, mussels in the mainstem were limited to sites around and downstream of the confluence with Styx Creek. Interestingly, Styx Creek flowed continually throughout the study period, while Nile Creek stopped flowing by mid-summer, suggesting that permanent dilution from the Styx provides a flow-on effect for the lower reach that the Nile does not. This underscores the importance of conserving the integrity and permanence of freshwater flows in drying climates, which presents a significant challenge given the growing pressure on these resources from human population growth (Cañedo-Argüelles et al., 2013).

While the persistence of *W. carteri* in the Kent catchment can largely be attributed to the refuge provided by fresh tributaries, the species is also clearly more robust than first reported. Mussels were already known to occur in the lower reach (Klunzinger et al., 2012a), which is surprising given that mean annual salinities often exceeded 1.6 g/L in the 1980s and 90s, reaching >2.5 g/L in 1987 and surpassing 2.3 g/L as recently as 2002. Similarly, the frequency and duration of extreme events regularly exceeded laboratory derived toxicity thresholds for the species. Despite this, the largest mussels (up to 80 mm SL) collected at the lower-most sites are likely to be 35-80 years of age (based on Klunzinger et al., 2014), suggesting that they persisted there throughout the salinity peak at the turn of the century. Laboratory derived salinity thresholds for invertebrates have been shown to be strong predictors of their distribution in the field (Kefford et al., 2004; Horrigan et al., 2007), nonetheless, some species in those



studies were still collected at salinities substantially higher than their LC<sub>50</sub>. The findings of the present study add support to recent findings of Ma (2018) that *W. carteri's* LC<sub>50</sub> and LC<sub>95</sub> thresholds may be substantially higher than previously reported when mussels are exposed to more natural high salinity conditions. Similarly, research in North America has shown that the salinity tolerance of *Lampsilis fasciola* glochidia is higher in natural surface water rather than reconstituted water used in many experiments (Gillis, 2011). The fact that the two lower-most sites had regular recruitment and among the highest densities in this study, while mussels were lost from other nearby sites in the lower reach, highlights the small-scale 'patchiness' that refuges may exhibit, and that mussel populations may appear robust even when they are in decline, depending on the spatial extent of the sites sampled.

Although salinity appears to be the primary driver of *W. carteri* decline their distribution is also limited by a range of other natural and anthropogenic factors (Klunzinger et al., 2015). In this study, mussels were common at freshwater tributary sites ranging in pH from 5.4 to 7.5; however, they were not detected at tributary sites where conditions were highly acidic (pH <4.5). Mollusks are often vulnerable to acidic conditions, in part because a high concentration of H<sup>+</sup> ions inhibits the uptake of calcium for shell building (Økland & Økland, 1986). Young mussels may be particularly susceptible, and laboratory experiments in North America have shown that one-week old juveniles of *Utterbackia imbecillis* and *Pyganodon cataracta* had a 96 hour LC<sub>50</sub> of pH 4.5, and that >50% died within 72 hours at pH 4 (Dimock & Wright, 1993). It is not known whether the high levels of acidity at some sites in the present study occur naturally, or indicate some form of disturbance. Southwestern Australia is highly impacted by surface water acidification, a phenomenon linked to rising saline groundwater and point-source discharges caused by soil excavation (Halse, Ruprecht, & Pinder., 2003; Degens et al., 2012). Numerous old mussel shells were detected at some acidic sites in this study, indicating their presence there in the past. Based on our data, if acidification of the upper part of Styx Creek is occurring, then the refuge provided by that tributary may have been reduced to <6 km of habitat.

#### *Salinity reversal and mussel recruitment*

Mussels are long-lived, slow-growing, and late to mature, so are unlikely to recover quickly from disturbance (Strayer et al., 2004), and there are very few studies on the recovery of mussel populations where they have been lost (Riccardi et al., 2016). In one of the few examples of recovery, Sietman et al. (2001) found that after mussels were extirpated from the Illinois River by urban waste pollution in the early 20<sup>th</sup> century, they did not begin recolonising until the 1980s. Similarly, Riccardi et al. (2016) found that *Unio elongatulus* was not detected in Lake Orta for almost 90 years after an industrial pollution

event wiped out most of the lakes biota in 1926. While other studies have found some evidence for invertebrate recovery following reductions in point-source salt pollution (Bathe & Coring, 2011), species diversity and other biological metrics remain diminished in those rivers, and the time to full recovery is not known (Arle & Wagner, 2013). Additionally, point-source salt discharges represent a different mechanism from the landscape scale salinity studied here (sensu Zipper et al., 2016). In this study, we found that *W. carteri* was extirpated from, and has recently begun to recolonise, two of the five mainstem sites where mussels were detected. Based on age-at-length measurements for other populations of this species, lengths of >65 mm SL are not reached for >15 years (Klunzinger et al., 2014, Benson et al., 2018). No mussels of that size were found at the two recolonisation sites, while individuals of 65-80 mm SL dominated the population structure further downstream. The inferred age of the largest mussels from the two recolonisation sites suggests that they recruited post-2002, coinciding with salinity reversal in the river (Figure 2). In combination, these results suggest that: (1) mussels in the lower reach of the river were approaching a tipping-point for extirpation before salinity began to reverse; and (2) recolonisation may begin relatively quickly when salinity is reversed.

While we believe that our results constitute the first evidence of recolonisation by mussels following salinity reversal, it is worth noting that recruitment may remain sporadic for some time. Young mussels (<54 mm SL) were common in the lower reach; however the two sites where recolonisation has occurred lacked a cohort in the 18-35 mm SL range, and no young-of-the-year were detected at any site. Although this could be due to difficulties in detecting smaller individuals or naturally sporadic recruitment (Hastie et al., 2000), it could also reflect annual fluctuations in salinity levels that have deviated from the overall trend of decline. For example, since 2012 the annual mean salinity has ranged from 0.69 to 1.69 g/L, with brief 5 g/L events each year from 2014 to 16 (Figure 2).

#### *Limits to mussel re-expansion*

Despite increased recruitment in the lower reach, *W. carteri* distribution in the Kent River is still limited, and it remains to be seen just how far upstream they can recolonise. One factor that may be particularly limiting to mussel recovery is evapoconcentration, which is a growing concern in med-regions due to increasing aridity and longer periods of no-flow (Robson et al., 2013). Evapoconcentration has been implicated in the total loss of salt-sensitive fish species in other parts of southwestern Australia (Beatty et al., 2011). As with many med-rivers, the Kent is experiencing reduced flows and it is expected that no-flow periods will increase by >4-months per year (Barron et al., 2012). During the study period, the mainstem stopped flowing by mid-autumn, causing the lower reach (including Kmid1) to become fresher

as it came under increasing influence of flow from Styx Creek. Above that point, salinity steadily increased (exceeding the LC<sub>50</sub> for the majority of the study period), and no mussels were detected. Although our data show that mussels have survived at higher salinities at lower-reach sites in the past, the durations were never so prolonged. These extended periods of elevated salinity constitute a chronic exposure scenario that has not been evaluated in laboratory trials thus far. Interestingly, salinity at sites above Kmid1 never exceeded the higher, more recent laboratory-derived LC<sub>50</sub> and LC<sub>95</sub> values for the species, which were conducted over 30 days (Ma, 2018). Future laboratory experiments should consider chronic exposure scenarios, using lower doses over longer durations, and incorporate natural surface water and environmentally relevant mixtures of ions (Gillis, 2011; Kunz et al., 2013). Alternatively, rather than single contaminant laboratory trials, an in-field mesocosm approach might be effective, although salinity will be confounded with other stressors such as temperature.

Another issue potentially limiting mussel re-expansion is the interruption of *W. carteri*'s lifecycle by intra-annual variability in salinity levels. In many of southwestern Australia's rivers, salinity is elevated in winter-spring due to the increase in saline runoff from the catchment, which coincides with the release of glochidia by *W. carteri* (Klunzinger et al., 2012b). While salinity is known to be particularly toxic to the younger life-stages of some mussels (Prosser et al., 2017), and to reduce glochidial attachment to host-fish (Blakeslee et al., 2013; Beggel & Geist, 2015), this remains a key knowledge gap for the conservation of many species. Similarly, saline reaches may present a barrier to dispersal of salt-sensitive host-fish as some species, such as the critically endangered *Galaxias truttaceus*, are restricted to freshwater refuges in saline rivers (Beatty et al., 2011; Morgan et al., 2016). This fish species is believed to have driven *W. carteri* recolonisation via a fish-ladder in a reach of the nearby Goodga River (Benson et al., 2018), and is one of the few highly mobile fish in the region that would be capable of long-distance dispersal in the Kent River. There has been some research on the effect of salinity on fish species distribution in southwestern Australia (Beatty et al., 2011); however, the impact this has on the connectivity of mussel populations is not well understood.

It is worth noting that while projected low rainfall scenarios will have negative effects on aquatic fauna by reducing freshwater inputs during summer base-flow (Beatty et al., 2011; Cañedo-Argüelles et al., 2013), the opposite may have also occurred here. Reduced saline-flow from the upper Kent River during summer-autumn has likely increased the freshening effect of Styx Creek on the lower reach in recent years. This may be in part responsible for the recent increase in mussel recruitment, and highlights the

unpredictability of outcomes arising from interactions between large-scale stressors such as salinity and climate change (Cañedo-Argüelles et al., 2013; Estevez et al., 2018).

### *Conclusions*

As one of the world's most heavily salinised landscapes (Halse, Ruprecht, & Pinder., 2003; Pettit et al., 2015), southwestern Australia is providing a growing number of examples of salinity reversal through improved catchment management (Mayer, Ruprecht, & Bari., 2005); however, reversing the impacts on freshwater biodiversity represents an additional challenge (Arle & Wagner, 2013; Cañedo-Argüelles et al., 2013; Herbert et al., 2015). Mussels are important ecosystem engineers and their global decline is having measurable effects on ecosystem processes (Vaughn, Atkinson, & Julian, 2015; Vaughn, 2018). The effects of mussel population recovery might also be expected to be detectable; however, this has not been assessed. Similarly, the outcomes of these engineering activities may change with the altered environmental context (*sensu* Spooner & Vaughn, 2012), as pre-disturbance salinity levels may not be reached for many years, if at all.

While mussel populations are expected to recover slowly from disturbance (Strayer et al., 2004), this study has found some evidence of rapid recolonisation following salinity reversal. Despite this, the extent of recovery is very limited, and further re-expansion may not be possible due to other unknown factors. More research into the capacity for natural recovery is necessary. Secondary salinity will overlap and interact with other stressors such as acidification and climate change, making the outcomes of management hard to predict (Degens et al., 2012; Cañedo-Argüelles et al., 2013). In light of this unpredictability, freshwater tributaries globally, and particularly those in med-regions, will become increasingly valuable as refuges for freshwater fauna. Discussion of refuges in highly seasonal ecosystems generally focuses on areas of permanent water during drought (e.g. Magoulick & Kobza, 2003; Hermoso, Ward, & Kennard., 2013); however, more frequent and severe storms events could increase the regularity of hyper-saline runoff events, so refuge at high rather than low flow should also be considered (Robson et al., 2013). Identifying appropriate conservation and restoration actions for maintaining the integrity and permanence of these refuges should be a priority for the management of freshwater biodiversity.

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## Chapter 5:

Evidence for multiple refugia and hotspots of genetic diversity for the only freshwater mussel in southwestern Australia



**Cover picture:** *Freshwater mussels from Gardner River in the Shannon Basin*

Benson, J.A., Stewart, B.A., Close, P.G. & Lymbery, A.J. (in review). Evidence for multiple refugia and hotspots of genetic diversity for the only freshwater mussel in southwestern Australia. *Aquatic Conservation: Marine and Freshwater Ecosystems*

## Abstract

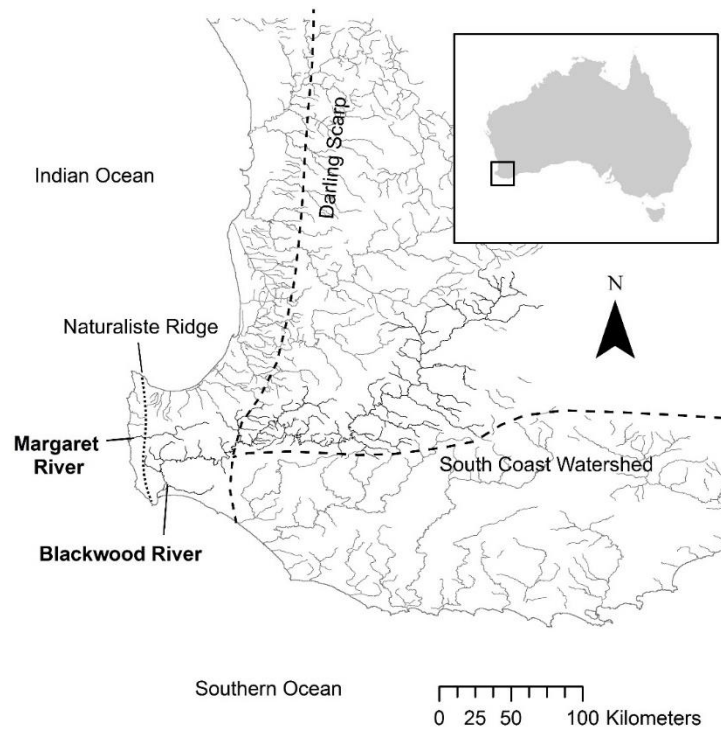
Intraspecific genetic diversity provides the evolutionary potential to adapt to changing environments and 'hotspots' of high intraspecific diversity are recognised as key targets for the conservation of threatened taxa. In southwestern Australia, intraspecific genetic diversity for mesic taxa is not uniformly distributed. Many species comprise highly divergent lineages with unique haplotypes as a result of contraction to refugia during historical cycles of aridity. Studies of the region's unique and ancient freshwater fauna have not had sufficient spatial resolution to describe the location of boundaries between lineages, and the identification of hotspots is lacking. This study explored the spatial distribution of intraspecific genetic diversity in the threatened freshwater mussel, *Westralunio carteri*. Mitochondrial DNA sequences for 164 specimens, sampled from all basins within the distribution of the species, were used to describe lineage boundaries and the location of hotspots, and to reconstruct historical demographics of this species. There was strong evidence for three subregions of mussel diversity based on the largely non-overlapping distributions of three evolutionary significant units (ESUs). Spatial and demographic analyses suggest these ESUs persisted through past arid cycles in separate refugia. The majority of haplotypes were unique to a single location, indicating limited connectivity among populations in recent times. Overall, diversity was higher in the south and southwestern parts of the distribution, although hotspots were identified throughout the region. Most notably, a significant hotspot in the southwestern corner has likely arisen through the overlap of lineages in a historical refugia. Conservation assessments often focus on the species as a whole, even though sublineages, hotspots and the threats faced are not evenly distributed across the species range. This study highlights that effective conservation of wide-ranging taxa may require independent management of multiple ESUs.

## Introduction

Intraspecific genetic diversity provides species with the evolutionary potential to adapt to changing environments and decreases the risk of extinction (Frankham, 1995; Saccheri et al., 1998; Hoffmann & Sgro, 2011; Lankau et al., 2011; Alberto et al., 2013). It is an integral component of biodiversity, and can alter community dynamics and ecosystem function through its influence on processes such as nutrient cycling and trophic cascades (Des Roches et al., 2018; Raffard et al., 2019). Intraspecific genetic diversity has been heavily impacted by human activities (Miraldo et al., 2016; Mimura et al., 2017), with a conservative estimate of 5.4%–6.5% loss within populations of wild organisms since the industrial revolution (Leigh et al., 2019). Despite its widely acknowledged importance and ongoing decline, intraspecific genetic diversity is often a neglected aspect of biodiversity conservation (Mimura et al., 2017).

‘Hotspots’ of intraspecific genetic diversity (areas of exceptionally high diversity) are increasingly recognised as key targets for species conservation and management (Petit et al., 2003; Canestrelli et al., 2010; Chiochio et al., 2021). Hotspots not only contain populations with greater evolutionary potential, but often have important environmental resources that may allow those populations to persist when facing stressful conditions (Canestrelli et al., 2014). Indeed, the generation of hotspots has been linked to persistence within environmentally stable refugia during past climate change (Hewitt, 2000; Canestrelli et al., 2010; Canestrelli et al., 2014; Nistelberger et al., 2014). Traditionally, this process has been described as a contraction to major refugia, where a panmictic, stable, and genetically diverse population is maintained (Hewitt, 2000). More recently, numerous authors have shown the process may actually involve ‘refugia-within-refugia’, whereby multiple isolated populations persist within the broader refugial area (Byrne, 2008; Canestrelli et al., 2010; Canestrelli et al., 2014; Chiochio et al., 2021). In this scenario, hotspots are ‘melting pots’ generated by (a) localised contraction and divergence of these populations and (b) subsequent admixture during re-expansion (Canestrelli et al., 2010). Under this model, priority areas for conservation might exist within the broader refugia; however, the spatial resolution of many phylogeographic studies is too coarse to pinpoint these areas and allow targeted allocation of resources (Zampiglia et al., 2019).

The Mediterranean-climate (med-) region of southwestern Australia (SWA; Figure 1) displays exceptional biodiversity (Hopper & Gioia, 2004; Rix et al., 2015), including many unique and ancient lineages of freshwater fauna (Davies & Stewart, 2013). Biogeographers have puzzled over the origins of



**Figure 1:** Location of southwestern Australia on the Australian continent (top right), and major topographical features subdividing the Southwest Drainage Division (based on Beard, 1999)

this biodiversity, as the ancient and weathered landscape lacks major barriers to dispersal that are typically the driver of speciation (Hopper & Gioia, 2004; Rix et al., 2015). For mesic taxa in the region, numerous phylogeographic studies have shown that intraspecific diversity is not uniformly distributed throughout the region, and that many species comprise highly divergent lineages as a result of contraction to separate refugia during early-mid Pleistocene arid cycling (Edwards, Roberts & Keogh, 2007; Edwards, Roberts & Keogh, 2008; Nistelberger et al., 2014). Within these primary lineages, a secondary pattern of highly localised haplotypes is also common in both widespread and restricted species, indicating more recent isolation of populations due to increasing aridification (Byrne, 2008; Byrne et al., 2011). The presence of a number of relictual plant and animal species on the South Coast has been linked to continued high rainfall in that area during arid phases, and suggests that this area may have acted as an important refugia at those times (Hopper, 1979; Wardell-Johnson & Horwitz, 1996; Roberts et al., 1997; Hopper & Gioia, 2004). Similarly, Nistelberger et al. (2014) found evidence of a significant refugial area on the South Coast based on high intraspecific diversity in the widespread shrub, *Calothamnus quadrifidus*. Ongoing aridification, combined with landuse change, secondary salinisation, and overexploitation of water resources, among other threats, has created an increasingly challenging

environment for both mesic and freshwater taxa (Robson et al., 2013; Pettit et al., 2015). There is an urgent need to better understand patterns of intraspecific diversity in both of those groups; however, research on taxa restricted to freshwater ecosystems is comparatively lacking (Davies & Stewart, 2013).

Despite subdued topography, rivers in SWA have long been known to group into distinct West Coast and South Coast Systems, with an additional System in the intermediate southwestern corner comprising the Blackwood Basin (Bettenay & Mulcahy, 2007) (Figure 1). Rivers on the West Coast flow from the Darling Plateau across the Darling Scarp to the Indian Ocean, constrained from the south by an extensive South Coast Watershed (Beard, 1999). The generally smaller rivers on the South Coast originate in the low elevations of that same watershed, flowing down the Ravensthorpe Ramp to the Southern Ocean (Cope, 1974; Beard, 1999). The Blackwood, in contrast to other South Coast rivers, originates far inland (~300 km) and flows west through a narrow gap between the South Coast Watershed and the Darling Scarp, before being deflected south by the Naturaliste Ridge to the region's southwestern corner (Beard, 1999). Another exception to these general groupings, Margaret River, emerges adjacent to the lower Blackwood River on the Blackwood Plateau, flowing west across the Naturalist Ridge into the 'Capes' region, where it enters the Indian Ocean along with a handful of other smaller drainages (Department of Water, 2007). Interestingly, phylogeographic studies on freshwater fish (Galeotti et al., 2015; Buckley et al., 2018), freshwater crayfish (Gouws, Stewart & Daniels, 2006; Gouws, Stewart & Daniels, 2010) and freshwater mussels (Klunzinger et al., 2021) have found evidence of genetic structuring into western and southern lineages, with additional lineages in either the Blackwood Basin or Margaret River. The apparent concordance between regional topography and freshwater phylogeography hints at the existence of distinct subregions of freshwater biodiversity in SWA. This would have significant implications for freshwater biodiversity conservation in the region; however, previous phylogeographic studies (also including Nguyen et al., 2002; Munasinghe, Murphy & Austin, 2003; Unmack et al., 2011) have not had sufficient spatial resolution (ie. at river or basin scale) to describe the location of boundaries between lineages, particularly in areas around the Capes and Blackwood Basin. Further, these studies have generally focused on structuring between lineages and populations, and there do not appear to be any instances where the distribution of intraspecific hotspots have been presented for wide-ranging freshwater taxa.

Freshwater mussels (*Bivalvia*, *Unionida*; hereafter 'mussels') are an ideal group for investigating intraspecific hotspots and historical refugia in freshwater ecosystems. They are slow to evolve, having a low nucleotide substitution rate, and so can be useful in reconstructing demographic events over long



time-spans (i.e. millions of years) (Zieritz et al., 2020). While mussels are highly vulnerable to environmental change (Lopes-Lima et al., 2018), particularly in med-regions (Benson et al., 2021), they are also important ecosystem engineers, providing important resources and improving habitat conditions for other taxa (Vaughn & Spooner, 2006; Aldridge, Fayle & Jackson, 2007; Limm & Power, 2011; Atkinson et al., 2013; Negishi et al., 2013). This being the case, areas that have served as refugia for mussels might also be important for the conservation of other taxa too. *Westralunio carteri* is the only recognised mussel in SWA (Klunzinger et al., 2015). Endemic to the region, *W. carteri* is widespread and often locally abundant (Klunzinger et al., 2015; Benson et al., 2018; Benson et al., 2019), but is listed as Vulnerable to Extinction on the IUCN Redlist due to contraction in range (Klunzinger & Walker, 2020). Recently, Klunzinger et al. (2021) found evidence of distinct western and southern lineages within *W. carteri* (WcI and WcII-III respectively), as well as additional substructuring that separates individuals from Margaret River (WcIII) from the rest of the southern lineage (WcII). The authors hypothesised that these three units had non-overlapping distributions, with WcI limited to West Coast basins north of the Capes, WcII limited to South Coast basins, and WcIII limited to the Capes, and proposed they be recognised as evolutionary significant units (ESUs) (Klunzinger et al., 2021). While the findings of Klunzinger et al. (2021) have clear implications for the conservation of this species, the study included few locations on the South Coast (3/8 basins) and only one location on the Capes, an area of potential overlap of the proposed ESUs (Figure 1). In order to maximise the conservation outcomes of this previous work, finer spatial resolution is needed.

The aim of the present paper is to describe the spatial distribution of *W. carteri* diversity in SWA at various scales, using a combination of newly collected and previously published genetic sequences. Given the lack of resolution in previous studies of freshwater taxa in the region, samples are incorporated from every basin within the known range of this species, and three specific hypotheses are tested:

1. *W. carteri* populations comprise three ESUs with non-overlapping distributions.
2. The WcII ESU will have the highest genetic diversity of the three ESUs due to long term stability on the South Coast.
3. There will be a prevalence of private haplotypes throughout SWA due to recent lack of connectivity among river basins.

Finally, the spatial distribution of genetic diversity within *W. carteri* is used to highlight priority areas for conservation and make inferences about the location of historical refugia in SWA.

## Methods

### ***Specimen collection***

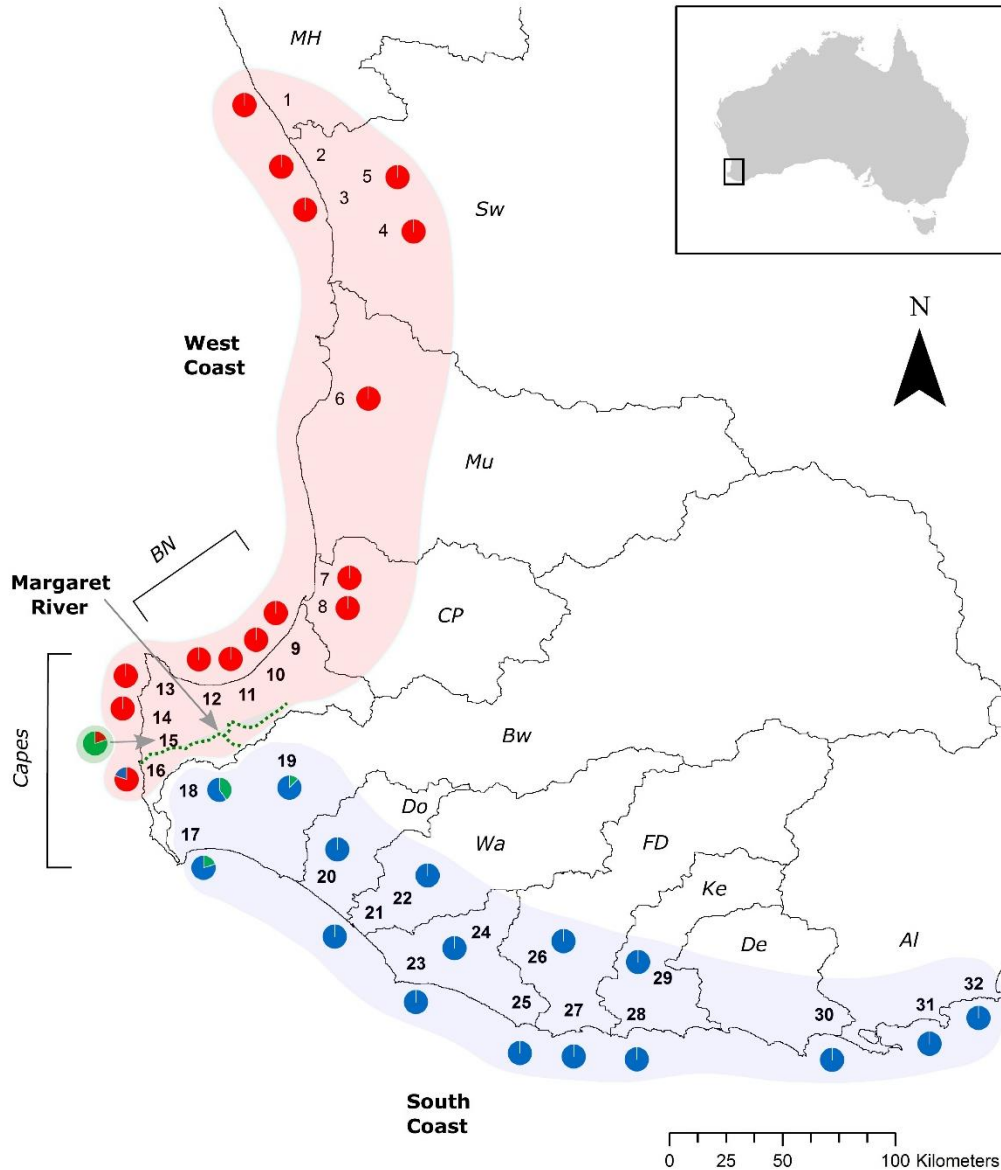
Fieldwork was conducted during the summer of 2019-20 in waterbodies (including rivers, creeks, and lakes) within the nine coastal drainage basins comprising the south and southwestern parts of the known distribution of *W. carteri* (Figure 2; Table 1). This area was targeted because it has been underrepresented in previous sampling of the species. In order to provide greater phylogeographic resolution at a potential boundary between lineages (Klunzinger et al., 2021) the Busselton Coast Basin was divided at the Naturaliste Ridge into (1) Busselton-North and (2) the Capes (Figure 2).

Searches were conducted in locations where mussels had previously been reported (e.g. Klunzinger et al., 2015; Benson et al., 2018; Benson et al., 2019), as well as other locations with habitat likely to support mussel populations. Sites were searched using visual and tactile methods for up to 1 hour per site. Mussels were not collected where >10 individuals could not be located within 10 minutes. In total, 120 mussels were collected from 24 sites ( $n = 5$  per site), with only one individual collected from any 1 m<sup>2</sup> of sediment. Mussels were stored on ice during transport to the laboratory, where a section of tissue was excised from the foot and stored in 100% ethanol. Sampling and site access was approved by The Department of Biodiversity, Conservation and Attractions (Regulation 17, Licence to take fauna for scientific purposes: No. 01-000037-1) and the Department of Fisheries (Research exemption number 3019).

### ***DNA extraction and analysis***

Nuclear (18S and 28S rDNA) and mitochondrial (COI and 16S rDNA) gene sequences were extracted from tissue samples for this study; however, the nuclear genes were found to have insufficient variation to be informative (all sequences were identical except one individual with two mutations in 28S) and only mitochondrial DNA sequences were subsequently used in genetic analyses. Extraction was performed by Helix Molecular Solutions with the Qiagen blood and tissue kit using instructions supplied by the manufacturer (QIAGEN, Hilden, Germany). The final elution volume was adjusted to 60µl, then repeated with a second elute of the same volume. The COI sequences (687 bp) were extracted using LCOI and HCO2 primers (Folmer et al., 1994) at a MgCl<sub>2</sub> concentration of 3mM and an annealing temperature of 48°C. The 16S sequences (517-518 bp) were extracted using 16Sar\_L\_myf and 16Sbr\_L\_myf primers (Lydeard, Mulvey & Davis, 1996) at a MgCl<sub>2</sub> concentration of 4mM and an annealing temperature of 50°C. Each 25µl PCR contained 0.25 µM of forward and reverse primer, 0.2 mM each dNTP, varied mM

MgCl<sub>2</sub>, 1 x PCR buffer, 0.4mg/ml BSA (Fisher Biotec, Western Australia) 0.05U *Taq* DNA Polymerase (Thermo Fisher Scientific, USA) and 3µl of DNA. Amplification was performed using an Eppendorf MasterCycler *epgradient S* (Eppendorf, Hamburg, Germany). Unpurified PCR products were sequenced by the Australian Genome Research Facility (AGRF) on an AB 3730x1 using BDT v3.1 reaction mix (Applied Biosystems, USA). All sequences were edited using the program Geneious 6.1.8 (<https://www.geneious.com>). These sequence data have been submitted to the GenBank databases under accession numbers provided in Table 1 ([www.ncbi.nlm.nih.gov/genbank](http://www.ncbi.nlm.nih.gov/genbank)). Accession numbers and extraction details for nuclear genes can be found in Table S1. An additional 46 COI and 12 16S *W. carteri* sequences were obtained from GenBank; however, one individual was removed from the dataset due to uncertainty over the collection site (accession numbers MT040670 (COI) and MT040067 (16S)), providing an initial dataset of 165 (COI) and 131 (16S) sequences in total.



**Figure 2:** Distribution of *Westralunio carteri* haplogroups (COI mtDNA) in southwestern Australia. Numbers show approximate site locations. Numbers in bold show sites where mussels were collected during this study; additional sequences were sourced from Genbank (see Table 1 for river/lake names and sample sizes). Pie charts show proportion of WcI (red), WcII (blue), and WcIII (green) haplotypes at each site. Basins are delineated according to the Australian Hydrological Geospatial Fabric (BOM/Aust gov: <https://data.gov.au/dataset/ds-dga-12937333-1f11-4167-a39b-6327794d4e83/details>). Basin names (in italics) correspond to Table 1. Note that the Busselton Coast Basin is divided into Capes and Busselton North (BN). Shaded areas show subregions as determined by AMOVA and SAMOVA (West Coast (red), South Coast (blue), and Margaret River (black/yellow dashed line))

**Table 1:** Sample locations for *Westralunio carteri* mtDNA (COI and 16S), with GenBank Accession numbers. Basin abbreviations and waterbody numbers correspond to Figure 2. Haplotypes marked NA were not included in the analysis. NB: Accession numbers and museum vouchers for samples collected during this study are still to be determined (listed as TBD)

| Location   |                     |                      | COI |           | 16S |           | ESU | Voucher        |
|------------|---------------------|----------------------|-----|-----------|-----|-----------|-----|----------------|
| Subregion  | Basin               | Waterbody            | Hap | Accession | Hap | Accession |     |                |
| West Coast | Moore-Hill (MH)     | Gingin Bk (1)        | 1   | MT040666  | -   | -         | Wcl | WAM<br>S82791  |
| "          | Swan Coast (SC)     | Marbling Bk (2)      | 2   | MT040671  | -   | -         | "   | WAM<br>S82790  |
| "          | "                   | Neerigen Bk (3)      | 3   | KP184917  | 1   | KP184870  | "   | UMMZ<br>304516 |
| "          | "                   | Wungong Bk (4)       | 5   | MT040656  | -   | -         | "   | WAM<br>S56225  |
| "          | "                   | "                    | 4   | MT040657  | -   | -         | "   | WAM<br>S56226  |
| "          | "                   | "                    | 4   | MT040658  | -   | -         | "   | WAM<br>S56229  |
| "          | "                   | Lk Leschenaultia (5) | NA  | MT040670  | NA  | MT040067  | NA  | WAM<br>S82739  |
| "          | "                   | "                    | 3   | KP184918  | 2   | KP184871  | "   | UMMZ<br>304517 |
| "          | Murray (Mu)         | Serpentine R (6)     | 7   | MT040664  | 1   | MT040065  | "   | WAM<br>S82779  |
| "          | "                   | "                    | 7   | MT040651  | -   | -         | "   | WAM<br>S56220  |
| "          | "                   | "                    | 7   | MT040652  | -   | -         | "   | WAM<br>S56221  |
| "          | "                   | "                    | 8   | MT040653  | -   | -         | "   | WAM<br>S56222  |
| "          | "                   | "                    | 6   | MT040654  | -   | -         | "   | WAM<br>S56223  |
| "          | "                   | "                    | 7   | MT040655  | -   | -         | "   | WAM<br>S56224  |
| "          | Collie-Preston (CP) | Collie R (7)         | 10  | MT040665  | 1   | MT040066  | "   | WAM<br>S82777  |
| "          | "                   | "                    | 6   | MT040628  | -   | -         | "   | WAM<br>S56210  |
| "          | "                   | "                    | 9   | MT040629  | -   | -         | "   | WAM<br>S56211  |
| "          | "                   | "                    | 11  | MT040630  | -   | -         | "   | WAM<br>S56212  |
| "          | "                   | "                    | 6   | MT040631  | -   | -         | "   | WAM<br>S56213  |
| "          | "                   | "                    | 6   | MT040632  | -   | -         | "   | WAM<br>S56214  |
| "          | "                   | Preston R (8)        | 6   | MT040646  | -   | -         | "   | WAM<br>S56215  |
| "          | "                   | "                    | 6   | MT040647  | -   | -         | "   | WAM<br>S56216  |
| "          | "                   | "                    | 6   | MT040648  | -   | -         | "   | WAM<br>S56217  |
| "          | "                   | "                    | 6   | MT040649  | -   | -         | "   | WAM<br>S56218  |

|                   |                         |                   |    |          |   |          |       |               |
|-------------------|-------------------------|-------------------|----|----------|---|----------|-------|---------------|
| "                 | "                       | "                 | 12 | MT040650 | - | -        | "     | WAM<br>S56219 |
| "                 | Busselton-North<br>(BN) | Capel R (9)       | 6  | TBD      | 1 | TBD      | "     | TBD           |
| "                 | "                       | "                 | 6  | "        | 1 | "        | "     | "             |
| "                 | "                       | "                 | 6  | "        | 3 | "        | "     | "             |
| "                 | "                       | "                 | 6  | "        | 1 | "        | "     | "             |
| "                 | "                       | "                 | 6  | "        | 1 | "        | "     | "             |
| "                 | "                       | Abba R (10)       | 6  | "        | 1 | "        | "     | "             |
| "                 | "                       | "                 | 6  | "        | 1 | "        | "     | "             |
| "                 | "                       | "                 | 6  | "        | 1 | "        | "     | "             |
| "                 | "                       | "                 | 6  | "        | 1 | "        | "     | "             |
| "                 | "                       | Ludlow R (11)     | 9  | "        | 1 | "        | "     | "             |
| "                 | "                       | "                 | 6  | "        | 1 | "        | "     | "             |
| "                 | "                       | "                 | 6  | "        | 1 | "        | "     | "             |
| "                 | "                       | "                 | 13 | "        | 4 | "        | "     | "             |
| "                 | "                       | "                 | 6  | "        | 1 | "        | "     | "             |
| "                 | "                       | Carbunup R (12)   | 1  | "        | 1 | "        | "     | "             |
| "                 | "                       | "                 | 6  | "        | 1 | "        | "     | "             |
| "                 | "                       | "                 | 6  | "        | 1 | "        | "     | "             |
| "                 | "                       | "                 | 6  | "        | 1 | "        | "     | "             |
| "                 | "                       | "                 | 6  | "        | 1 | "        | "     | "             |
| "                 | Capes                   | Wilyabrup Bk (13) | 9  | "        | 1 | "        | "     | "             |
| "                 | "                       | "                 | 9  | "        | 1 | "        | "     | "             |
| "                 | "                       | "                 | 9  | "        | 1 | "        | "     | "             |
| "                 | "                       | "                 | 9  | "        | 1 | "        | "     | "             |
| "                 | "                       | "                 | 9  | "        | 1 | "        | "     | "             |
| "                 | "                       | Ellensbrook (14)  | 9  | "        | 1 | "        | "     | "             |
| "                 | "                       | "                 | 9  | "        | 1 | "        | "     | "             |
| "                 | "                       | "                 | 9  | "        | 1 | "        | "     | "             |
| "                 | "                       | "                 | 9  | "        | 1 | "        | "     | "             |
| "                 | "                       | "                 | 9  | "        | 1 | "        | "     | "             |
| Margaret<br>River | "                       | Margaret R (15)   | 14 | "        | 1 | "        | "     | "             |
| "                 | "                       | "                 | 16 | "        | 5 | "        | WcIII | "             |
| "                 | "                       | "                 | 6  | "        | 1 | "        | WcI   | "             |
| "                 | "                       | "                 | 15 | "        | 5 | "        | WcIII | "             |
| "                 | "                       | "                 | 17 | "        | 5 | "        | "     | "             |
| "                 | "                       | "                 | 15 | MT040641 | 5 | MT040060 | "     | WAM<br>S56235 |
| "                 | "                       | "                 | 19 | MT040642 | 6 | MT040061 | "     | WAM<br>S56236 |
| "                 | "                       | "                 | 15 | MT040643 | - | -        | "     | WAM<br>S56237 |

|             |                |                    |    |          |    |          |       |               |
|-------------|----------------|--------------------|----|----------|----|----------|-------|---------------|
| "           | "              | "                  | 15 | MT040644 | -  | -        | "     | WAM<br>S56238 |
| "           | "              | "                  | 18 | MT040645 | -  | -        | "     | WAM<br>S56239 |
| West Coast  | "              | Boodjiup Bk (16)   | 9  | TBD      | 1  | TBD      | Wcl   | TBD           |
| "           | "              | "                  | 9  | "        | 1  | "        | "     | "             |
| "           | "              | "                  | 9  | "        | 1  | "        | "     | "             |
| "           | "              | "                  | 9  | "        | 1  | "        | "     | "             |
| "           | "              | "                  | 23 | "        | 7  | "        | WcII  | "             |
| South Coast | Blackwood (Bw) | Scott R (17)       | 24 | "        | 7  | "        | "     | "             |
| "           | "              | "                  | 25 | "        | 7  | "        | "     | "             |
| "           | "              | "                  | 24 | "        | 7  | "        | "     | "             |
| "           | "              | "                  | 19 | "        | 5  | "        | WcIII | "             |
| "           | "              | "                  | 26 | "        | 7  | "        | WcII  | "             |
| "           | "              | Chapman Bk (18)    | 20 | "        | 5  | "        | WcIII | "             |
| "           | "              | "                  | 28 | "        | 9  | "        | WcII  | "             |
| "           | "              | "                  | 27 | "        | 7  | "        | "     | "             |
| "           | "              | "                  | 28 | "        | 9  | "        | "     | "             |
| "           | "              | "                  | 21 | "        | 8  | "        | WcIII | "             |
| "           | "              | St John's Bk (19)  | 24 | "        | 10 | "        | WcII  | "             |
| "           | "              | "                  | 24 | "        | 7  | "        | "     | "             |
| "           | "              | "                  | 29 | "        | 7  | "        | "     | "             |
| "           | "              | "                  | 22 | "        | 11 | "        | WcIII | "             |
| "           | "              | "                  | 29 | "        | 7  | "        | WcII  | "             |
| "           | "              | "                  | 24 | MT040660 | 7  | MT040062 | "     | WAM<br>S66164 |
| "           | "              | "                  | 24 | MT040659 | -  | -        | "     | WAM<br>S82773 |
| "           | "              | "                  | 27 | MT040661 | -  | -        | "     | WAM<br>S66165 |
| "           | Donnelly (Do)  | Donnelly R (20)    | 23 | TBD      | 7  | TBD      | "     | TBD           |
| "           | "              | "                  | 23 | "        | 7  | "        | "     | "             |
| "           | "              | "                  | 23 | "        | 7  | "        | "     | "             |
| "           | "              | "                  | 23 | "        | 7  | "        | "     | "             |
| "           | "              | "                  | 23 | "        | 7  | "        | "     | "             |
| "           | Warren (Wa)    | Yeagerup Lake (21) | 30 | "        | 12 | "        | "     | "             |
| "           | "              | "                  | 31 | "        | 7  | "        | "     | "             |
| "           | "              | "                  | 33 | "        | 7  | "        | "     | "             |
| "           | "              | "                  | 32 | "        | 7  | "        | "     | "             |
| "           | "              | "                  | 32 | "        | 7  | "        | "     | "             |
| "           | "              | Warren R (22)      | 33 | "        | 7  | "        | "     | "             |
| "           | "              | "                  | 34 | "        | 7  | "        | "     | "             |
| "           | "              | "                  | 32 | "        | 7  | "        | "     | "             |
| "           | "              | "                  | 32 | "        | 7  | "        | "     | "             |

|   |                     |                |    |          |    |          |    |            |
|---|---------------------|----------------|----|----------|----|----------|----|------------|
| " | "                   | "              | 32 | "        | 7  | "        | "  | "          |
| " | Shannon (Sh)        | Gardner R (23) | 35 | "        | 8  | "        | "  | "          |
| " | "                   | "              | 36 | "        | 8  | "        | "  | "          |
| " | "                   | "              | 35 | "        | 8  | "        | "  | "          |
| " | "                   | "              | 35 | "        | 8  | "        | "  | "          |
| " | "                   | "              | 35 | "        | 8  | "        | "  | "          |
| " | "                   | Shannon R (24) | 37 | "        | 7  | "        | "  | "          |
| " | "                   | "              | 34 | "        | 7  | "        | "  | "          |
| " | "                   | "              | 32 | "        | 7  | "        | "  | "          |
| " | "                   | "              | 38 | "        | 13 | "        | "  | "          |
| " | "                   | "              | 38 | "        | 13 | "        | "  | "          |
| " | "                   | Inlet (25)     | 37 | "        | 7  | "        | "  | "          |
| " | "                   | "              | NA | "        | NA | "        | NA | "          |
| " | "                   | "              | 37 | "        | 7  | "        | "  | "          |
| " | "                   | "              | 27 | "        | 14 | "        | "  | "          |
| " | "                   | "              | 37 | "        | 7  | "        | "  | "          |
| " | Frankland-Deep (FD) | Deep R (26)    | 39 | "        | 8  | "        | "  | "          |
| " | "                   | "              | 39 | "        | 8  | "        | "  | "          |
| " | "                   | "              | 37 | "        | 8  | "        | "  | "          |
| " | "                   | "              | 40 | "        | 7  | "        | "  | "          |
| " | "                   | "              | 39 | "        | 8  | "        | "  | "          |
| " | "                   | Walpole R (27) | 37 | "        | 8  | "        | "  | "          |
| " | "                   | "              | 37 | "        | 8  | "        | "  | "          |
| " | "                   | "              | 37 | "        | 8  | "        | "  | "          |
| " | "                   | "              | 37 | "        | 8  | "        | "  | "          |
| " | "                   | "              | 37 | "        | 8  | "        | "  | "          |
| " | Kent (Ke)           | Bow R (28)     | 37 | "        | 8  | "        | "  | "          |
| " | "                   | "              | 43 | "        | 8  | "        | "  | "          |
| " | "                   | "              | 35 | "        | 8  | "        | "  | "          |
| " | "                   | "              | 42 | "        | 8  | "        | "  | "          |
| " | "                   | "              | 41 | "        | 8  | "        | "  | "          |
| " | "                   | Kent R (29)    | 35 | "        | 8  | "        | "  | "          |
| " | "                   | "              | 35 | "        | 8  | "        | "  | "          |
| " | "                   | "              | 35 | "        | 8  | "        | "  | "          |
| " | "                   | "              | 35 | "        | 8  | "        | "  | "          |
| " | "                   | "              | 35 | "        | 8  | "        | "  | "          |
| " | "                   | "              | 37 | MT040636 | 8  | MT040058 | "  | WAM S56205 |
| " | "                   | "              | 44 | MT040637 | 8  | MT040059 | "  | WAM S56206 |
| " | "                   | "              | 35 | MT040638 | -  | -        | "  | WAM S56207 |



|   |                       |                    |    |          |    |          |   |                 |
|---|-----------------------|--------------------|----|----------|----|----------|---|-----------------|
| " | "                     | "                  | 35 | MT040639 | -  | -        | " | WAM<br>S56208   |
| " | "                     | "                  | 37 | MT040640 | -  | -        | " | WAM<br>S56209   |
| " | "                     | "                  | 37 | MT040667 | -  | -        | " | WAM<br>S82758.1 |
| " | "                     | "                  | 34 | MT040668 | -  | -        | " | WAM<br>S82758.2 |
| " | Denmark Coast<br>(De) | Marbellup Bk (30)  | 37 | TBD      | 8  | TBD      | " | TBD             |
| " | "                     | "                  | 37 | "        | 8  | "        | " | "               |
| " | "                     | "                  | 37 | "        | 8  | "        | " | "               |
| " | "                     | "                  | 37 | "        | 8  | "        | " | "               |
| " | "                     | "                  | 37 | "        | 8  | "        | " | "               |
| " | Albany Coast<br>(Al)  | Goodga R (31)      | 35 | "        | 8  | "        | " | "               |
| " | "                     | "                  | 35 | "        | 8  | "        | " | "               |
| " | "                     | "                  | 35 | "        | 8  | "        | " | "               |
| " | "                     | "                  | 35 | "        | 8  | "        | " | "               |
| " | "                     | "                  | 41 | MT040669 | -  | -        | " | WAM<br>S82756   |
| " | "                     | "                  | 35 | MT040633 | -  | -        | " | WAM<br>S56200   |
| " | "                     | "                  | 35 | MT040634 | -  | -        | " | WAM<br>S56202   |
| " | "                     | "                  | 35 | MT040635 | -  | -        | " | WAM<br>S56203   |
| " | "                     | Waychinicup R (32) | 34 | TBD      | 7  | TBD      | " | TBD             |
| " | "                     | "                  | 34 | "        | 7  | "        | " | "               |
| " | "                     | "                  | 34 | "        | 7  | "        | " | "               |
| " | "                     | "                  | 46 | "        | 15 | "        | " | "               |
| " | "                     | "                  | 34 | "        | 7  | "        | " | "               |
| " | "                     | "                  | 34 | MT040662 | 7  | MT040063 | " | WAM<br>S66127   |
| " | "                     | "                  | 45 | MT040663 | 7  | MT040064 | " | WAM<br>S66128   |

### ***Data management***

Individual alignments were constructed using the full dataset for each mtDNA gene using the ClustalW accessory application in Bioedit 7.2.5 (Hall, 1999). Sequences within each alignment were inspected and trimmed to equal length in MEGA X version 10.1.8 (Kumar et al., 2018). Far fewer sequences were available for 16S than for COI, so the two genes were analysed separately rather than concatenating. This also allowed comparison between the results for the two genes, which evolve at different rates (see Zieritz et al. (2020) for recently determined rates). All sequences were assessed to determine if they were female (f-type) mtDNA. Many bivalves, including Unionida, exhibit doubly uniparental inheritance (DUI), whereby the transmission of mitochondrial DNA to offspring is not exclusively from the maternal line (Hoeh et al., 1996). While female mussels contain f-type mtDNA in all of their tissue, male mussels contain f-type mtDNA in their somatic tissue and a divergent male (m-type) mtDNA in their gonads and gametes (Hoeh et al., 1996; Breton et al., 2007). The degree of divergence between f- and m-type mtDNA within bivalve species varies depending on the gene assessed and can range from 20 to >50% (uncorrected nucleotide p-distance) (Breton et al., 2007; Doucet-Beaupré et al., 2010). A threshold of 20% divergence (i.e. the difference between any two sequences in the dataset) was used to identify potentially m-type mtDNA. A BLAST search was conducted on potentially m-type sequences, and those that were more closely matched to m-type than f-type mtDNA were removed from the analysis. Based on this approach, one individual was removed (uncorrected p-distance = 21.88%) as a BLAST search found that the closest match for 16S was the m-type genome of fellow hyriid, *Echyriddella menziesii*. All other p-distances were <3.80%.

### ***Phylogeny and genetic diversity***

Multiple approaches were employed to discern phylogenetic patterns and describe genetic diversity for each gene. Maximum likelihood (ML) and Bayesian inference (BI) trees were constructed in MEGA X version 10.1.8 (Kumar et al., 2018) and MrBayes version 3.2.7a (Ronquist et al., 2012) respectively, using alignments reduced to unique haplotypes in DNAsP v6 (Rozas et al., 2017). The MEGA software identified the appropriate substitution models as HKY+G (Hasegawa, Kishino & Yano, 1985) for COI, and the Tamura 3-parameter model (Tamura, 1992) for 16S following methods outlined in Hall (2013). The ML analysis was performed with 10,000 bootstrap replicates under the general settings of the selected models. The BI analysis was performed using the selected substitution models with 20,000 MCMC generations sampling every 1,000 generations. For each analysis, reciprocal monophyly of the WcI and WcII-III lineages identified by Klunzinger et al. (2021) was confirmed (i.e. using a threshold of 100% for

ML bootstrap and BI posterior probabilities), and then each lineage was used as the outgroup for the other lineage. This allowed us to utilise a more recent ancestor than is otherwise available for *W. carteri*, which is highly divergent even from other Australian hyriids (Graf et al., 2015), thereby providing greater resolution in more recent branches. Support values from ML bootstrapping and BI posterior probabilities were defined as full (100%), strong (>95%), moderate (>70), and weak (0-70%). ML and BI trees were edited for publication in FigTree v1.4.4 (<http://tree.bio.ed.ac.uk/software/figtree/>) and Inkscape v1 (<https://inkscape.org>).

As tree-building methods may not appropriately capture haplotypic relationships in instances of shallow divergence (Posada & Crandall, 2001), haplotype networks were constructed for both COI and 16S, using the alignments of all individuals in TCS 1.21 (Clement, Posada & Crandall, 2000). The networks were edited for publication in tcsBU (Múrias dos Santos et al., 2016). DnaSP v6 (Rozas et al., 2017) was used to identify unique haplotypes, and to calculate DNA polymorphism summary statistics for each gene (haplotype richness (h), haplotype diversity (Hd), nucleotide diversity (Pi), sequence diversity (k), and number of segregating sites (S)).

### ***Population structure and spatial diversity***

The COI gene was used to test for spatial population structure and to determine the number and location of different mussel ‘subregions’. The COI gene was chosen rather than 16S because (1) it provided larger sample sizes and allowed more rivers to be included in the analysis, and (2) it has a faster mutation rate (Zieritz et al., 2020) so is more likely to detect evidence of structuring. Individuals from various locations in the Swan Coast Basin were pooled for analysis due to small sample sizes ( $n = 1-3$ ) and Gingin Brook was excluded ( $n = 1$ ).

Analysis of molecular variance (AMOVA) was performed in Arlequin 3.5.2.2 (Excoffier & Lischer, 2010) to test for hierarchical patterns of genetic variation. Populations (individual rivers) were assigned to groups (i.e. potential subregions) inferred from patterns in the haplotype networks, and from previous phylogeographic studies of SWA freshwater taxa (Gouws, Stewart & Daniels, 2006; Gouws, Stewart & Daniels, 2010; Galeotti et al., 2015; Buckley et al., 2018; Klunzinger et al., 2021). Firstly, three models were tested proposing two subregions; (1) West coast/Capes vs South coast, (2) West coast vs South coast/Capes, and (3) West coast/Capes (minus Margaret River) vs South coast/Margaret river. Secondly, three models were tested proposing three subregions; (1) West coast vs Capes vs South coast, (2) West coast/Capes (minus Margaret River) vs Margaret River vs South coast, (3) West coast/Capes (minus Margaret River) vs Margaret River/Blackwood vs South coast (minus Blackwood). Finally, for the model

that maximised the among group variance, an AMOVA was conducted separately for each identified subregion, grouping the constituent rivers by basin. All AMOVAs were based on pairwise differences and significance was assessed using 10,000 permutations of the data.

In order to objectively verify the results of the a priori AMOVAs, spatial analysis of molecular variance (SAMOVA; Dupanloup, Schneider & Excoffier, 2002) was also used to define subregion boundaries. The SAMOVA approach uses a simulated annealing procedure to define geographically homogeneous populations while maximising the among group variance ( $F_{CT}$ ) for a given number of groups ( $K$ ) (Dupanloup, Schneider & Excoffier, 2002). Separate SAMOVAs were run for groups (i.e. potential subregions) ranging in number from 2-14, with each SAMOVA based on 100 simulated annealing steps. The best model was chosen following Zúñiga-Vega et al. (2014), by plotting the difference in  $F_{CT}$  for each increase in the number of groups, and identifying the maximum point (Appendix 2, Figure S1).

Pairwise  $F_{ST}$  values were calculated in Arlequin (Excoffier & Lischer, 2010) to show genetic differentiation among the subregions defined by the best fitting model of the AMOVA/SAMOVA approach, as well as among basins and among rivers in SWA overall. A heatmap was constructed in R to display the pairwise  $F_{ST}$  values (R Core Team, 2018). Summary statistics for DNA polymorphism (haplotype richness ( $h$ ), haplotype diversity ( $Hd$ ), and nucleotide diversity ( $Pi$ )) were also calculated for subregions and basins using DnaSP v6 (Rozas et al., 2017).

### **Demographic history**

In order to search for evidence of population growth or stability within *W. carteri* lineages, tests for departures from neutral equilibrium assumptions were performed on the COI and 16S genes. A range of complementary tests were undertaken to account for their various strengths and weaknesses. Fu's  $F_s$  (Fu, 1997) and  $R_2$  (Ramos-Onsins & Rozas, 2002) were selected because they perform well with large and small sample sizes respectively, while Tajima's  $D$  (Tajima, 1989) was selected as a comparatively conservative approach. Mismatch distributions (MMDs) were plotted for the three COI lineages to compare the observed frequency of differences between pairs of sequences to that expected under a model of population growth. The models described in each plot were tested using Harpending's raggedness index ( $R_g$ ; Harpending et al., 1993) with non-significant results used as an indicator of an expanding population. Statistical significance of all tests was assessed in DnaSP v6 (Rozas et al., 2017), using 10,000 coalescent simulations given theta.

Bayesian skyline plots (BSPs) (Drummond et al., 2005) were constructed to depict historical demographic fluctuations and time to most recent common ancestor (TMRCA) for each COI lineage. The BSP models were created in BEAST 2.6.2 (Bouckaert et al., 2019) using 20 million MCMC generations and sampling every 1,000 steps. A strict clock model prior was selected along with the HKY+G model as with the phylogenetic analysis, and we used the above mentioned substitution rate. The BSPs were constructed in Tracer v1.7.1 (Rambaut et al., 2018) after discarding the initial 10% of trees as burn-in and assessing parameter convergence in the same software.

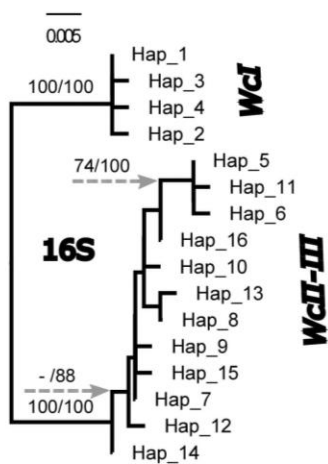
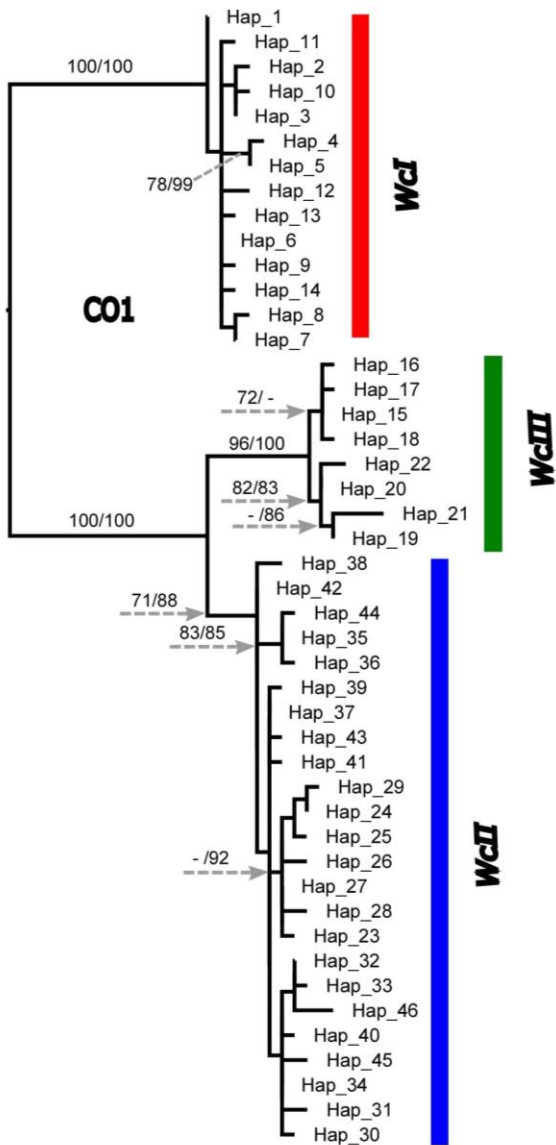
## Results

F-type mtDNA gene fragments for COI and 16S were successfully obtained for 119 of the 120 individuals collected for this study. Together with samples obtained from Genbank, the COI alignment comprised 164 sequences of 549 base pairs (bp), and the 16S alignment comprised 130 sequences of 461 bp. Overall, the COI alignment had 46 distinct haplotypes, compared to 16 for the 16S alignment.

### ***Phylogeny and genetic diversity***

Phylogenetic reconstructions for both COI and 16S fully supported reciprocal monophyly of the two primary lineages (WcI and WcII-III) as bootstrap values (BS) and posterior probabilities (BPP) were all 100% (Figure 3). The ML and BI trees displayed identical topologies so only the ML trees are displayed. Within the WcII-III lineage there was strong (BS = 96%) and full (BPP = 100%) support for monophyly of WcIII based on the COI sequences; support for monophyly of WcII was moderate (BS = 71%; BPP = 88%). There was some evidence of additional substructuring within both WcII and WcIII, in particular with the separation of samples from the Blackwood Basin within both of those groups (see BS and BPP values in Figure 3). For the 16S sequences, moderate (BS = 74%) and full (BPP = 100%) support was found for monophyly of WcIII; support for monophyly of WcII was either weak (BS = 63%) or moderate (BPP = 88%).

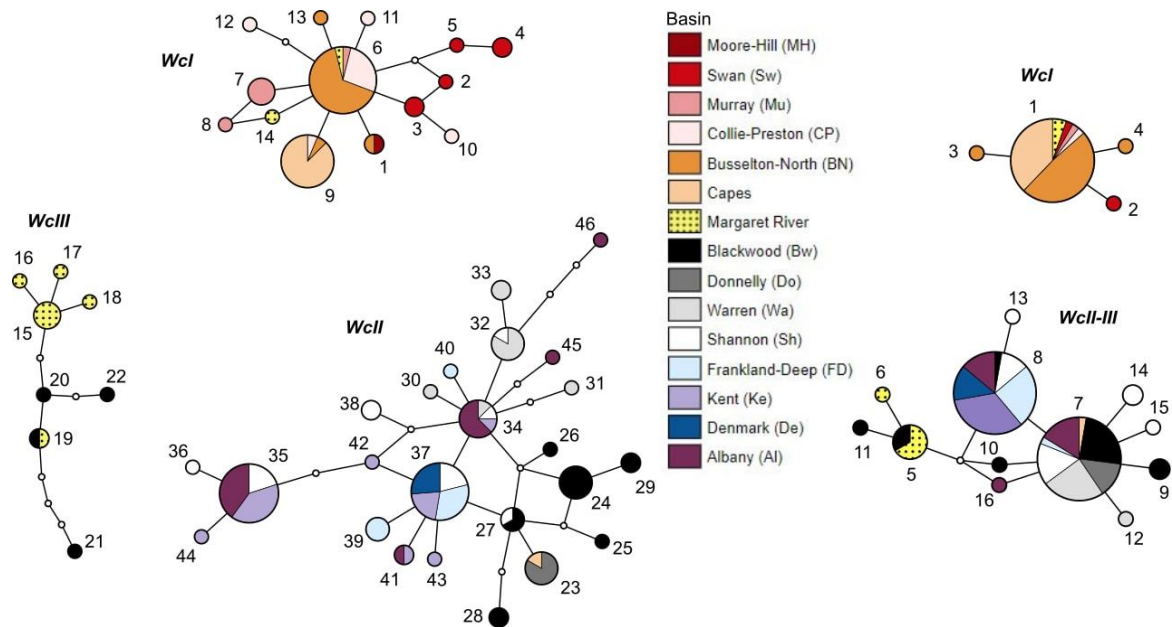
The sequences resolved into three haplogroups based on COI, and two groups based on 16S (Figure 4). For COI haplotypes, WcI and WcII were separated by at least 23 mutations, while WcIII was separated from WcI and WcII by 27 and 10 mutations respectively. For 16S, WcI and WcII-III were separated by at least 11 mutations. The greatest haplotype diversity was found in WcII and WcIII (COI) and WcII-III (16S) (Figure 4; Table 2). For the COI gene, WcI comprised 14 haplotypes, WcII comprised 24 haplotypes, and WcIII comprised 8 haplotypes (Figure 4; Table 2). For 16S, WcI and WcII-III comprised 4 and 12 haplotypes respectively (Figure 4; Table 2). The COI haplotype network displayed a more exploded



**Figure 3:** Maximum likelihood (ML) tree showing relationships among *Westralunio carteri* mtDNA haplotypes and clades (COI, top; 16S, bottom). Branch values are ML bootstrap/Bayesian posterior probabilities (BS/BPP) respectively. Any BS values <70 and BPP <85 are not shown. Colours relate to clade distribution in Figure 2. Scale bar is for both trees

layout than that of 16S, reflecting its faster evolutionary rate and the presence of more recent mutations. All polymorphism summary statistics were lowest in WcI for both COI and 16S (Table 2).

For COI, there was little overlap in the spatial distribution of the three haplogroups, with WcI found exclusively at locations on the West Coast and Capes, and WcII found almost exclusively at locations on the South Coast (all but one individual, which came from Boodjiup Creek on the Capes). However, WcIII haplotypes were found in both Margaret River (Capes) and the three rivers sampled in the Blackwood Basin (on the South Coast) (Figure 2). Additionally, two samples from Margaret River belonged to WcI. The origin of samples within the 16S network reflected that of COI, and WcI was clearly separated while WcII and WcIII merged into the WcII-III lineage (Figure 4).



**Figure 4:** Haplotype networks (TCS) for *Westralunio carteri* COI (three haplogroups left of legend) and 16S (two haplogroups right of legend) mtDNA. Colours correspond to basin of origin (haplotypes with a single colour are private to that basin). Abbreviated basin names correspond to Figure 2 and individual haplotypes numbers correspond to Figure 3. Note that the Capes does not include Margaret River haplotypes. Hollow points are unobserved haplotypes

**Table 2:** DNA polymorphism summary statistics and neutrality tests for *Westralunio carteri* haplogroups (COI above, 16S below). *h*, haplotype richness; *Hd*, haplotype diversity (SD); *Pi*, nucleotide diversity (SD); *k*, sequence diversity; *S*, number of segregating sites. For neutrality tests (last three columns) alpha was set at 0.02 for Fu's *F<sub>s</sub>*, and at 0.05 for Tajima's *D* and Ramos-Onsins and Rozas *R<sub>2</sub>* (Significant *P* values are in bold)

|     | <i>Haplogroup</i> | <i>n</i> | <i>h</i> | <i>Hd (SD)</i>   | $\pi$ (SD)       | <i>k</i> | <i>S</i> | <i>F<sub>s</sub> (P)</i>  | <i>D (P)</i>             | <i>R<sub>2</sub> (P)</i> |
|-----|-------------------|----------|----------|------------------|------------------|----------|----------|---------------------------|--------------------------|--------------------------|
| COI | WcI               | 60       | 14       | 0.743<br>(0.044) | 0.002<br>(0.000) | 1.26     | 12       | -8.889<br><b>(0.000)</b>  | -1.594<br><b>(0.026)</b> | 0.053<br>(0.059)         |
|     | WcIII             | 12       | 8        | 0.894<br>(0.078) | 0.005<br>(0.001) | 2.97     | 12       | -2.598<br>(0.046)         | -1.061<br>(0.162)        | 0.115<br>(0.058)         |
|     | WcII              | 92       | 24       | 0.894<br>(0.018) | 0.006<br>(0.000) | 3.044    | 27       | -10.598<br><b>(0.001)</b> | -1.354<br>(0.066)        | 0.054<br>(0.094)         |
|     | All COI           | 164      | 46       | 0.932<br>(0.009) | 0.028<br>(0.001) | 15.406   | 61       | -2.566<br>(0.323)         | 1.064<br>(0.888)         | 0.125<br>(0.937)         |
| 16S | WcI               | 40       | 4        | 0.146<br>(0.075) | 0.000<br>(0.000) | 0.15     | 3        | -3.884<br><b>(0.000)</b>  | -1.716<br><b>(0.001)</b> | 0.088<br>(0.202)         |
|     | WcII-III          | 90       | 12       | 0.672<br>(0.032) | 0.002<br>(0.000) | 1.069    | 10       | -6.141<br><b>(0.006)</b>  | -1.189<br>(0.113)        | 0.053<br>(0.134)         |
|     | All 16S           | 130      | 16       | 0.760<br>(0.017) | 0.014<br>(0.001) | 6.442    | 23       | 2.815<br>(0.824)          | 1.325<br>(0.930)         | 0.138<br>(0.955)         |

### **Population structure**

The AMOVA tests showed strong support for genetic structuring in the region, consistent with patterns observed in the phylogenetic trees and haplotype networks (Figures 2-4). All results for models proposing 2-3 subregions were highly significant (Table 3). The optimal model was for three subregions: (1) West coast/Capes (minus Margaret River), (2) Margaret River, and (3) South coast, with 84.27% of the variation explained by these subregions (See model v in Table 3). From here, these subregions are referred to as the West Coast, Margaret River, and South Coast subregions. This model was also the most parsimonious according to the SAMOVA (84.22%;  $P < 0.000$ ). Finally, the AMOVAs by basin for the West Coast and South Coast subregions (models vii and viii respectively; Table 3) found that most of the variance was explained at the subregional level (i.e. within all rivers) (68.09 and 56.07 respectively;  $P < 0.000$  for both). Interestingly, the variation explained among rivers within basins was also significant for the South Coast (29.53%;  $P = 0.004$ ) but not the West Coast (0.01%;  $P = 0.808$ ). No AMOVA was performed for the Margaret River subregion as it comprises just one population.



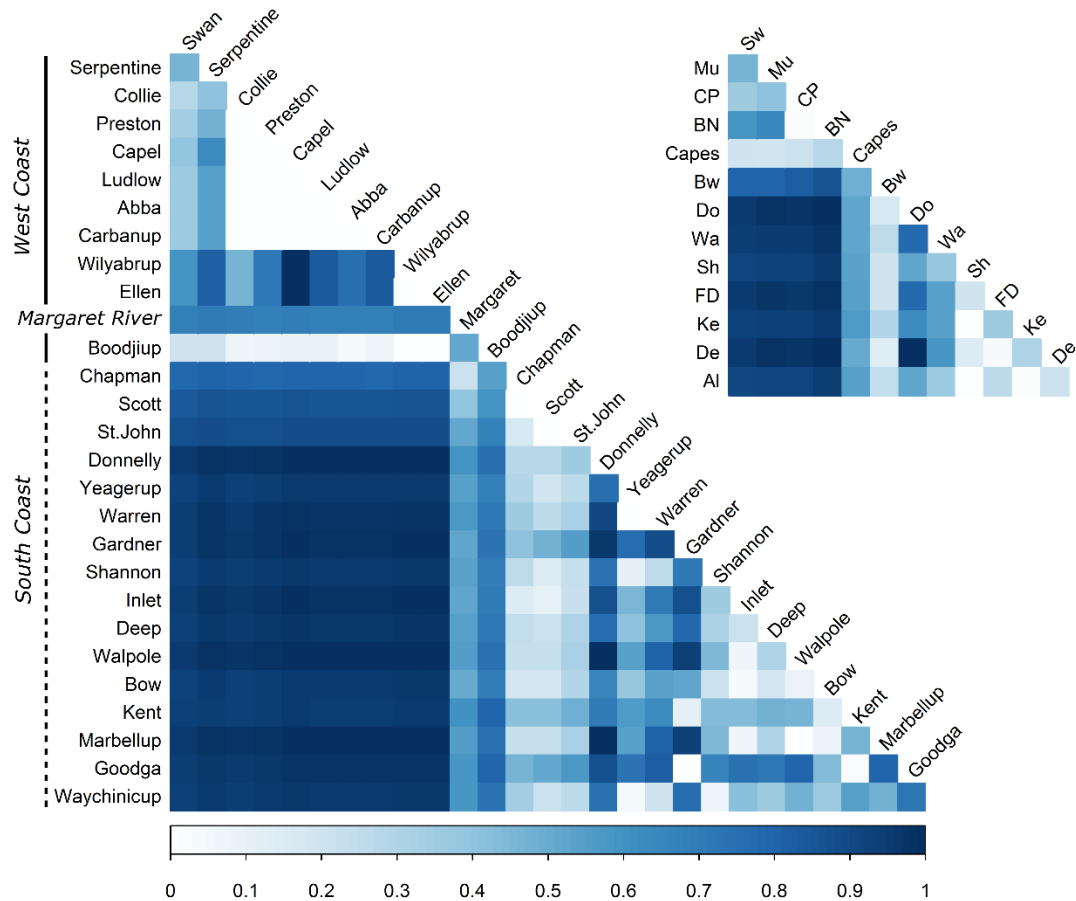
**Table 3:** Hierarchical analysis of molecular variance (AMOVA) in *Westralunio carteri* COI mtDNA. Analyses i-iii group populations (i.e. rivers) into one of two potential subregions; analyses iv-vi group populations into one of three potential subregions; analyses vii-viii group populations into basins within subregions described in model v

| Model and source of variation   | d.f. | s.s.    | Variance components | Variation % | Fixation index | P       |
|---|------|---------|---------------------|-------------|----------------|---------|
| <b>i. (1) West Coast/Capes - (2) South Coast</b>  |      |         |                     |             |                |         |
| Between subregions  | 1    | 781.93  | 9.72                | 76.47       | Fct = 0.76473  | < 0.000 |
| Among rivers within subregions  | 26   | 279.82  | 1.62                | 12.78       | Fsc = 0.54333  | < 0.000 |
| Within all rivers   | 135  | 184.35  | 1.37                | 10.74       | Fst = 0.89256  | < 0.000 |
| Total   | 162  | 1246.10 | 12.71               |             |                |         |
| <b>ii. (1) West Coast - (2) South Coast/Capes</b>   |      |         |                     |             |                |         |
| Between subregions  | 1    | 588.77  | 9.01                | 67.88       | Fct = 0.67882  | < 0.000 |
| Among rivers within subregions  | 26   | 472.98  | 2.90                | 21.83       | Fsc = 0.67983  | < 0.000 |
| Within all rivers   | 135  | 184.35  | 1.37                | 10.28       | Fst = 0.89717  | < 0.000 |
| Total   | 162  | 1246.10 | 13.28               |             |                |         |
| <b>iii. (1) West Coast/Capes (minus Margaret River) - (2) South Coast/Margaret River</b>                                  |      |         |                     |             |                |         |
| Between subregions  | 1    | 853.65  | 11.32               | 81.84       | Fct = 0.81842  | < 0.000 |
| Among rivers within subregions  | 26   | 208.10  | 1.14                | 8.28        | Fsc = 0.45607  | < 0.000 |
| Within all rivers   | 135  | 184.35  | 1.37                | 9.88        | Fst = 0.90123  | < 0.000 |
| Total   | 162  | 1246.10 | 13.83               |             |                |         |
| <b>iv. (1) West Coast - (2) Capes - (3) South Coast</b>   |      |         |                     |             |                |         |
| Among subregions  | 2    | 842.60  | 8.91                | 77.07       | Fct = 0.77068  | < 0.000 |
| Among rivers within subregions  | 25   | 219.15  | 1.29                | 11.12       | Fsc = 0.48483  | < 0.000 |
| Within all rivers   | 135  | 184.35  | 1.37                | 11.81       | Fst = 0.88186  | < 0.000 |
| Total   | 162  | 1246.10 | 11.56               |             |                |         |
| <b>v. (1) West Coast/Capes (minus Margaret River) - (2) Margaret River - (3) South Coast</b>                              |      |         |                     |             |                |         |
| Among subregions  | 2    | 938.05  | 10.71               | 84.27       | Fct = 0.84266  | < 0.000 |
| Among rivers within subregions  | 25   | 123.70  | 0.63                | 4.99        | Fsc = 0.31738  | < 0.000 |
| Within all rivers   | 135  | 184.35  | 1.37                | 10.74       | Fst = 0.89259  | < 0.000 |
| Total   | 162  | 1246.10 | 12.71               |             |                |         |
| <b>vi. (1) West Coast/Capes (minus Margaret River) - (2) Margaret River/Blackwood - (3) South Coast (minus Blackwood)</b> |      |         |                     |             |                |         |
| Among subregions  | 2    | 912.16  | 8.88                | 80.36       | Fct = 0.8036   | < 0.000 |
| Among rivers within subregions  | 25   | 149.59  | 0.81                | 7.29        | Fsc = 0.37097  | < 0.000 |
| Within all rivers   | 135  | 184.35  | 1.37                | 12.35       | Fst = 0.87646  | < 0.000 |
| Total   | 162  | 1246.10 | 11.05               |             |                |         |
| <b>vii. West Coast/Capes (minus Margaret River) basins only</b>   |      |         |                     |             |                |         |
| Among basins  | 4    | 19.795  | 0.37748             | 31.9        | Fct = 0.31901  | 0.001   |
| Among rivers within basins  | 6    | 4.838   | 0.00013             | 0.01        | Fsc = 0.00016  | 0.808   |
| Within all rivers   | 47   | 37.867  | 0.80567             | 68.09       | Fst = 0.31912  | < 0.000 |
| Total   | 57   | 62.5    | 1.18328             |             |                |         |
| <b>viii. South Coast basins only</b>  |      |         |                     |             |                |         |
| Among basins  | 7    | 60.392  | 0.30883             | 14.4        | Fct = 0.14401  | 0.074   |
| Among rivers within basins  | 8    | 38.679  | 0.63332             | 29.53       | Fsc = 0.34502  | 0.004   |
| Within all rivers   | 79   | 94.982  | 1.2023              | 56.07       | Fst = 0.43934  | < 0.000 |
| Total   | 94   | 194.053 | 2.14445             |             |                |         |

**Table 4:** Diversity data for *Westralunio carteri* mtDNA (COI left, 16S right) grouped spatially by subregion (in italics) and by river basin (basin abbreviations correspond with Figure 2). The West Coast subregion comprises rivers within basins from Moore-Hill to the Capes, minus Margaret River. The South Coast subregion comprises all rivers within basins from Blackwood to Albany. n = sample size, h = haplotype richness, Hd = haplotype diversity (and SD),  $\pi$  = nucleotide diversity (and SD), k = average number of nucleotide differences, S = number of segregating sites, and P = number of Parsimony informative sites. Note: Margaret River is also included in the Capes basin

| Grouping              | COI |    |               |               |        |    |    | 16S |    |               |               |       |    |    |
|-----------------------|-----|----|---------------|---------------|--------|----|----|-----|----|---------------|---------------|-------|----|----|
|                       | n   | h  | Hd (SD)       | $\pi$ (SD)    | k      | S  | P  | n   | h  | Hd (SD)       | $\pi$ (SD)    | k     | S  | P  |
| <i>West Coast</i>     | 59  | 14 | 0.749 (0.044) | 0.004 (0.002) | 2.191  | 37 | 8  | 39  | 5  | 0.197 (0.085) | 0.002 (0.001) | 0.821 | 16 | 0  |
| <i>Margaret River</i> | 10  | 7  | 0.867 (0.107) | 0.021 (0.008) | 11.444 | 33 | 29 | 7   | 3  | 0.667 (0.160) | 0.017 (0.006) | 7.905 | 17 | 16 |
| <i>South Coast</i>    | 95  | 28 | 0.901 (0.018) | 0.008 (0.001) | 4.129  | 43 | 28 | 84  | 11 | 0.638 (0.032) | 0.002 (0.000) | 0.878 | 9  | 5  |
| Moore-Hill (MH)       | 1   | 1  | -             | -             | -      | -  | -  | 0   | -  | -             | -             | -     | -  | -  |
| Swan (Sw)             | 6   | 4  | 0.867 (0.129) | 0.004 (0.001) | 2.267  | 4  | 4  | 2   | 2  | 0.667 (0.314) | 0.001 (0.001) | 1.000 | 1  | 0  |
| Murray (Mu)           | 6   | 3  | 0.600 (0.215) | 0.001 (0.001) | 0.667  | 2  | 0  | 1   | 1  | -             | -             | -     | -  | -  |
| Collie-Preston (CP)   | 11  | 5  | 0.618 (0.164) | 0.002 (0.001) | 1.091  | 6  | 0  | 1   | 1  | -             | -             | -     | -  | -  |
| Busselton-North (BN)  | 20  | 4  | 0.284 (0.128) | 0.001 (0.000) | 0.300  | 3  | 0  | 20  | 3  | 0.195 (0.115) | 0.000 (0.000) | 2.000 | 2  | 0  |
| Capes                 | 25  | 9  | 0.677 (0.098) | 0.028 (0.004) | 15.380 | 40 | 31 | 22  | 4  | 0.455 (0.115) | 0.014 (0.003) | 6.597 | 17 | 16 |
| Blackwood (Bw)        | 18  | 10 | 0.882 (0.063) | 0.014 (0.003) | 7.908  | 28 | 20 | 16  | 6  | 0.683 (0.120) | 0.003 (0.001) | 1.483 | 5  | 4  |
| Donnelly (Do)         | 5   | 1  | 0.000 (0.000) | 0.000 (0.000) | 0.000  | 0  | 0  | 5   | 1  | 0.000 (0.000) | 0.000 (0.000) | 0.000 | 0  | 0  |
| Warren (Wa)           | 10  | 5  | 0.756 (0.130) | 0.003 (0.001) | 1.422  | 5  | 2  | 10  | 2  | 0.200 (0.154) | 0.000 (0.000) | 0.200 | 1  | 0  |
| Shannon (Sh)          | 14  | 7  | 0.857 (0.065) | 0.005 (0.001) | 2.659  | 8  | 5  | 14  | 5  | 0.758 (0.084) | 0.002 (0.000) | 1.044 | 4  | 2  |
| Frankland-Deep (FD)   | 10  | 3  | 0.600 (0.131) | 0.002 (0.001) | 0.867  | 3  | 1  | 10  | 2  | 0.200 (0.154) | 0.000 (0.000) | 0.200 | 1  | 0  |
| Kent (Ke)             | 17  | 7  | 0.750 (0.092) | 0.004 (0.000) | 2.044  | 7  | 3  | 12  | 1  | 0.000 (0.000) | 0.000 (0.000) | 0.000 | 0  | 0  |
| Denmark (De)          | 5   | 1  | 0.000 (0.000) | 0.000 (0.000) | 0.000  | 0  | 0  | 5   | 1  | 0.000 (0.000) | 0.000 (0.000) | 0.000 | 0  | 0  |
| Albany (Al)           | 16  | 5  | 0.683 (0.091) | 0.005 (0.001) | 3.000  | 11 | 4  | 12  | 3  | 0.621 (0.087) | 0.002 (0.000) | 0.697 | 2  | 1  |

Paiwise  $F_{ST}$  values among subregions were similarly high between West Coast-Margaret River (0.840) and West Coast-South Coast (0.877), but were much lower between Margaret River-South Coast (0.655), and all results were significant ( $P < 0.000$ ). Pairwise  $F_{ST}$  values at river- and basin-scale were generally lower when comparing among West Coast rivers/basins and among South Coast rivers/basins than they were when comparing locations between the two subregions (Figure 5), further supporting the AMOVA results. Within the Capes,  $F_{ST}$  values for Margaret River were lower when compared to South Coast rivers than to West Coast rivers, while the inverse was true for all other rivers on the Capes.

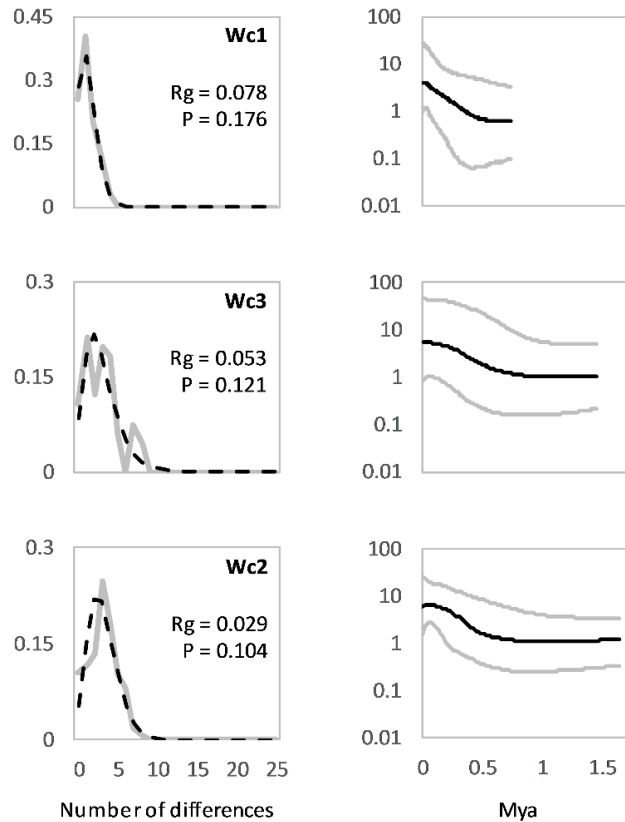


**Figure 5:** Heatmap matrix of pairwise  $F_{ST}$  values among rivers (bottom left) and basins (top right) for *Westralunio carteri* COI mtDNA. Basins are arranged from north-south on the West Coast and west-east on the South Coast. All rivers have five or more samples except Inlet River ( $n = 4$ ). Moore-Hill Basin/Gingin Brook are not included due to small sample size

The spatial structuring and relative diversities of *W. carteri* haplogroups effected the distribution of intraspecific diversity within each region. Haplotype diversity for both genes was higher in the South Coast and Margaret River than in the West Coast (Table 4). Despite this, the Swan Coast Basin on the West Coast had among the highest Hd values, along with the Blackwood and Shannon Basins on the South Coast. Higher COI diversity in Margaret River and the Blackwood Basin was driven by the presence of two haplogroups. When considering the Capes as a whole (i.e. Wilyabrup Brook, Ellensbrook Brook, Margaret River, and Boodjiup Brook), Hd was relatively low (COI = 0.677; 16S = 0.455) as 14/15 individuals collected from outside of Margaret River shared a single Wc1 haplotype for both genes (Table 1). The Donnelly and Denmark Basins each had a single haplotype for both genes, although only five individuals were collected from these basins. There was a prevalence of private haplotypes, with the majority being found in a single basin for both COI (76.1%) and 16S (75%) (see Figure 4). This was also the case for all individual COI haplogroups (WcI = 78.6%; WcII = 70.1%; WcIII = 87.5) and 16S haplogroups (WcI = 75%; WcII-III = 75%).

### ***Demographic history***

A signature of demographic expansion was detected by neutral equilibrium tests, mismatch distributions (MMDs) and Bayesian skyline plots (BSPs). Fu's  $F_s$  was strongly negative and significant for WcI ( $P < 0.001$ ) and WcII/WcII-III ( $P < 0.01$ ) for both COI and 16S, and Tajima's D was also significant for WcI for COI ( $P < 0.05$ ) and 16S ( $P < 0.001$ ) (Table 2). There was no evidence of expansion in WcIII. No significant results were found for Ramos-Onsins and Rozas  $R_2$  for either gene. Mismatch distributions for the COI gene were not significantly different to that expected under a model of population growth for any of the three haplogroups (raggedness index;  $P = 0.1$  or higher for all) (Figure 6). The multi-model mismatch distribution of WcIII may indicate that it comprises two independent populations, which could mask signals of expansion when using neutrality tests. The BSPs detected demographic expansion within all three haplogroups (Figure 6); WcII and WcIII appear to have began a gradual expansion ~0.75 Mya, with growth accelating ~0.5 Mya, while WcI appears to have grown rapidly from ~0.5 Mya. The WcI haplogroup appears to be much more recent in origin, with the TMRCA ancestor estimated (median and 95% HPD) at 0.75 Mya (0.23 – 1.65), compared to 1.47 Mya (0.57 – 2.82), and 1.66 (0.74 – 2.95) for WcIII, and WcII respectively.



**Figure 6:** Mismatch distributions for *Westralunio carteri* COI haplogroups (left). Distributions show the frequency of differences between pairs of sequences. The observed distribution (grey line) is compared to the distribution expected under a model of population growth (black dashes). Harpending's Raggedness (Rg) and P values are shown for each plot; Bayesian skyline plots of demographic history (right). Population size is expressed in terms of the effective population size per generation. Black lines are median estimates and grey lines show 95% highest posterior density estimates

## Discussion

Identifying hotspots of intraspecific diversity and areas of historical refugia is important for the conservation of imperiled taxa (Petit et al., 2003; Canestrelli et al., 2010; Chiochio et al., 2021). Mussels in med-regions are more imperiled and face a wider range of threats than other mussels globally, but phylogeographic research effort on this group has not been equal among these regions (Benson et al., 2021). This paper is the first comprehensive phylogeographic study encompassing all basins within the distribution of mussels in SWA, and the first such study for any wide-ranging freshwater taxon in the region. Consistent with the findings of Klunzinger et al. (2021), the present analysis of *Westralunio carteri* mtDNA confirmed two primary lineages (WcI and WcII-III), and the the existence of at least three ESUs overall (i.e. WcI, WcII, and WcIII). The largely non-overlapping distribution of the these ESUs

corresponds to three distinct subregions (West Coast, South Coast, and Margaret River) of genetic diversity for the species. Furthermore, spatial and demographic analyses suggest historical contraction of these ESUs to separate refugia during aridification, with subsequent re-expansion into their present ranges. Of the three ESUs, WcII and WcIII had the highest genetic diversity; however, the prevalence of private haplotypes within all three ESUs suggests limited connectivity among populations in recent times, and current persistence within multiple localised refugia. Areas of high diversity were identified within each subregion: most notably, Margaret River and the Blackwood Basin appear to be significant hotspots for mussel diversity in SWA. These findings highlight that effective conservation of wide-ranging taxa may require independent management of multiple ESUs.

Although the hypothesis that WcI, WcII, and WcIII do not overlap in distribution was not fully supported, the three ESUs appear to co-occur only rarely (Figure 2). The WcI ESU was only found on the West Coast, including Margaret River, while WcII was almost entirely restricted to the South Coast. There was only one instance of overlap between WcI and WcII, at Boodjiup Brook in the south of the Capes. The third ESU, WcIII, was unique in that it was common in two rivers located in separate subregions. Based on a more limited dataset, Klunzinger et al. (2021) hypothesised that WcIII was restricted to waterbodies to the west of the Naturaliste Ridge (i.e. the Capes), but surprisingly this ESU was not detected in Capes rivers outside of Margaret River in the present study, and these other rivers each contained a single WcI haplotype. Instead, WcIII was found extensively in the Blackwood Basin, indicating connectivity between Margaret River and the western end of the South Coast subregion, either currently or in the recent past. While the Blackwood Basin and Margaret River share the WcIII ESU, haplotypes from WcII were restricted to the Blackwood Basin, suggesting that connectivity between the two areas is currently limited. Further, the COI phylogeny of *W. carteri* suggests that haplotypes collected in the Blackwood Basin, whether WcII or WcIII, form monophyletic groups within those ESUs. The congruence of this pattern in two separate ESUs suggests that the Blackwood Basin is not only isolated from Margaret River, but also from other South Coast basins to the east, due to the inconspicuous boundary of the South Coast Watershed (Beard, 1999).

The hypothesis, that WcII would have higher genetic diversity than the other two ESUs reflecting historical climate stability on the South Coast, was only partly supported. Genetic diversity was also high for WcIII, while being much lower for WcI, suggesting that the Margaret River and the South Coast populations are older than those on the West Coast. This is supported by the more exploded layout of the the West Coast ESU, WcI, which is characteristic of recent demographic expansion following a

population bottleneck. Further, the TMRCA estimates for WcII and WcIII (~1.5ma) were approximately twice that of WcI (~750k), indicating that WcI populations on the West Coast experienced a period of decline and then re-expansion at a time when the South Coast and Margaret River were relatively stable. The TMRCA estimates of each ESU fall within the early- to mid-Pleistocene, consistent with the phylogeography of other taxa in the region (Byrne, 2008; Rix et al., 2015). Interestingly, the more recent origin of WcI during the mid-Pleistocene, is presumably associated with a climate shift to cycles of aridification, that has also been associated with the isolation and divergence of other taxa in mesic and freshwater refugia (Byrne, 2008; Rix et al., 2015). The BSPs presented here suggest that each ESU has undergone demographic expansion from ~500k until recently. For WcII and WcIII, this appears to have begun after a period of relative stability, while for WcI expansion occurred shortly after the TRMCA.

The hypothesis, that there would be a prevalence of private haplotypes in SWA river basins, was supported; moreover, the majority of haplotypes for both genes were only detected in a single river (Appendix 2, Tables S2 and S3). While there are a number of widespread haplotypes on the West Coast and South Coast, giving an impression of panmixia within those subregions, the prevalence of private haplotypes suggests that connectivity has been low in recent times, even between neighbouring rivers. For WcII on the South Coast, these widespread haplotypes are generally surrounded by private haplotypes, creating a complex of star-like structures within the haplogroup (Figure 4). This pattern is consistent with a sequence of events beginning with allopatry in multiple refugia (i.e. refugia-within-refugia), followed by admixture during range expansion, and subsequent re-isolation resulting in ongoing allopatry. Interestingly, AMOVA for South Coast basins found significant variation among rivers within the same basin; however, this was not the case on the West Coast. One possible explanation for this lower connectivity on the South Coast is that rivers in that area are generally small, while the larger rivers on the West Coast extend far inland onto the Darling Plateau. The headwaters of larger rivers may be more likely to come into contact, particularly during wetter interglacial periods, as suggested for frogs in the region (Edwards, Roberts & Keogh, 2007).

### *Conservation implications*

The discovery of three distinct subregions of mussel diversity in SWA has implications for conservation, as management strategies based on studies from one subregion may not be broadly transferable over the entire range of the species. Most of the work on *W. carteri* has been conducted on populations from the West Coast, including the identification of host fish (Klunzinger et al., 2012), salinity tolerances (Klunzinger et al., 2015), and behavioural response to drought (Lymbery et al., 2020). Similar studies are

now required for populations from the South Coast and Margaret River if they are to be managed appropriately. Additionally, the distribution of human populations and landuse practices vary across SWA, so each ESU may be exposed to different primary threats. For example, the West Coast is highly urbanised compared to the rest of the region, while South Coast rivers are impacted by salinised flows from the agricultural inland, and Margaret River and the creeks of the Capes have been extensively altered and utilised for viticulture and agriculture. The species is listed as Vulnerable based on a 49% reduction in distribution (Klunzinger & Walker, 2020), but a recovery plan has not been put in place as the current conservation advice is believed to provide sufficient direction for management (Threatened Species Scientific Committee, 2017). It is not known whether this observed decline is specific to one or more of the three ESUs. Further, the limited extent of occurrence of each ESU, particularly WcIII, suggests that the current listing might be inadequate. In order to maximise conservation efforts for *W. carteri*, each ESU requires independent management.

This study has identified significant areas for the conservation of mussels. These areas may also be important for freshwater biodiversity in general, as mussels provide valuable resources for other taxa, including algae (Atkinson et al., 2013), invertebrates (Vaughn & Spooner, 2006; Aldridge, Fayle & Jackson, 2007), and fish (Limm & Power, 2011; Negishi et al., 2013). Further, as mussels are highly vulnerable to environmental change (Lopes-Lima et al., 2018), areas that act as refugia for this group may play similar role for co-occurring species. The southwestern corner of SWA is clearly a hotspot that should be prioritised for the conservation of *W. carteri*. Margaret River and the Blackwood Basin are both 'melting pots' of mussel genetic diversity, with each containing representatives from two ESUs, and in combination, all three ESUs are represented in this small area. Both locations, but most notably the Blackwood Basin, contain numerous divergent haplotypes within single ESUs which also suggests long-term persistence within refugia. As WcIII almost certainly evolved in isolation from the other ESUs in one or both of these locations, and as it is not known to occur anywhere else, both locations should be considered equally important for the conservation and management of this species. These locations are known to have significant conservation value for freshwater biodiversity. For example, Margaret River is home to a number of unique or rare lineages of crayfish (Munasinghe, Murphy & Austin, 2003) and fish (Allen, Beatty & Morgan, 2017; Buckley et al., 2018), while the Blackwood Basin has the highest richness of freshwater fish species in SWA (Morgan et al., 2014). Due to limited information on intraspecific diversity in the region, it is not known whether *W. carteri* diversity is typical of freshwater biodiversity in this area. Additional studies on wide-ranging taxa are needed in order for a comparative phylogeography of freshwater biodiversity to be undertaken.



A number of other areas were notable for their high genetic diversity despite having a single ESU. Diversity in these areas was driven by divergent haplotypes within either WcI or WcII, so they are likely to be important areas for the conservation of those ESUs. On the central South Coast, the Shannon Basin had diversity values on par with those in Margaret River and the Blackwood Basin. The Shannon Basin has been identified as an area of high conservation value for other freshwater taxa (Sutcliffe, 2003), and is projected to be a significant refugia for freshwater fish under future climate scenarios (Stewart, Ford, & Benson, in review). Given the essential role that fish play as hosts for the development and dispersal of larval *W. carteri* (Klunzinger et al., 2012; Benson et al., 2018) the Shannon Basin may take on additional importance for WcII. Similarly, the Swan Coast was among the most diverse basins in this study despite being located on the West Coast, and COI phylogenies found moderate-strong support for a sublineage comprising individuals collected from Wungong Brook in the Swan Coast Basin (Figure 3; Table 1). Importantly, the spatial distribution of WcI haplotypes on the West Coast is characteristic of spatial expansion after contraction to refugia, with higher diversity in the northern half of the subregion and progressively lower diversity to the south. The larger rivers in that area are more typical of refugia than the small, genetically depauperate creeks sampled towards the south. The Collie-Preston, Murray, and Swan Coast had the highest COI diversity among West Coast basins, which may indicate long-term persistence of those populations. As WcI is restricted to the West Coast subregion, identifying the exact location should be a priority for its conservation. Additional samples, particularly from the Swan Coast Basin, could help to pinpoint such an area.

### *Conclusions*

Mussels in SWA comprise at least three, largely non-overlapping ESUs of a single species, and all three appear to have little opportunity to expand beyond the boundaries of their current distribution. They are likely to have differences in biology and ecology, as well as in the threats they face. In order for *W. carteri* to be effectively conserved, each ESU requires independent management. The apparently limited connectivity at the river and basin scale decreases the likelihood of recolonisation from outside sources if a population is lost. Better understanding of this sub-structuring is vital for future rearing programs or even translocations. The level of recent connectivity between populations could be determined using faster evolving genes and microsatellites. Pinpointing the exact location of refugia within each subregion will allow better management and protection of these areas, for mussels and freshwater biodiversity in general. Additional sampling of the northern West Coast, particularly within the Swan Coast Basin, is needed in order to fully describe the spatial distribution of intraspecific genetic diversity in that area.

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Chapter 6:  
General conclusions



*Cover picture: Secret location on the south coast of Western Australia*

## Overview and key findings

The reliance of human populations on freshwater resources has driven an unparalleled decline of biodiversity in freshwater ecosystems (Dudgeon et al., 2006; Reid et al., 2019; Tickner et al., 2020). In Mediterranean (med-) climate regions, these ecosystems often present naturally harsh conditions for freshwater taxa due to the characteristic seasonality of river flows, which includes high-flow in winter and low- or no-flow in summer (Aschmann, 1973; Gasith & Resh, 1999). This already stressful state has been exacerbated by intense anthropogenic development and subsequent disturbance in all med-regions (Cooper et al., 2013; Robson et al., 2013). Freshwater biodiversity in these regions is likely to be subjected to increasing pressure from a range of existing and emerging threats (Bonada & Resh, 2013), so there is an urgent need to better understand key factors for species conservation and the potential for restoration.

Freshwater mussels (Bivalvia, Unionida; hereafter 'mussels') provided an interesting opportunity to assess the conservation and potential for restoration of freshwater biodiversity within a med-region context. Mussel species have a suite of environmental characteristics that make them vulnerable to anthropogenic disturbance, and the group is experiencing an alarming global decline (Lopes-Lima et al., 2018; Ferreira-Rodriguez et al., 2019). At the same time, mussel populations are unlikely to recover quickly from these declines, due to their complex life cycle, slow growth rates, and long time to sexual maturity (Strayer et al., 2004). Concern over the plight of mussels is heightened because they are also key components of many freshwater ecosystems (Vaughn & Hakenkamp, 2001). Large and long-lived, they often congregate in densely clumped beds, which can contribute significantly to the biomass of freshwater ecosystems (Vaughn & Hakenkamp, 2001; Strayer et al., 2004; Vaughn, 2010). Further, mussels are recognised as important ecosystem engineers for the various functional roles they play, including the improvement of habitat conditions through filter feeding and bioturbation, as well as the provision of physical habitat and nutrients for benthic foodwebs (Vaughn & Hakenkamp, 2001; Aldridge, Fayle & Jackson, 2007; Atkinson & Vaughn, 2015). Given the global decline of mussels and the challenging conditions for freshwater biodiversity within med-regions, this thesis aimed to assess the plight of mussels in med-regions (med-mussels), and used *Westralunio carteri* as a case study in southwestern Australia to determine the potential for populations to recover following decline.

This research has demonstrated that the mussel fauna found in med-regions are characterised by low species richness, often with a single species per river, and that med-mussels are more imperiled than other mussel species globally (Chapter 2). Despite the extreme level of imperilment of med-mussels,

there is the potential to reverse population declines through the mitigation of threats at both localised (Chapter 3) and catchment scales (Chapter 4). Further, mussels have responded to the threat of aridification in the past by retreating to climate refugia throughout the landscape, followed by re-expansion when conditions were once again favourable (Chapter 5). These multiple lines of evidence for the resilience of med-mussels to contemporary and historical threats provides a degree of hope for the prospects of this group. In combination, four main implications for the conservation and restoration of mussels in med-regions arise from this thesis.

## **Implications and future research**

The first implication is that the low species richness of mussels within med-regions increases rather than diminishes the importance of this group, as there is little or no redundancy within these ecosystems if a species is lost. There is increasing awareness that biodiversity, whether at the genetic, species or functional group level, is involved in the maintenance ecological function and stability (Cardinale et al., 2012). Intact, well-functioning ecosystems are likely to be better able to respond to disturbance, whereas species-loss may increase instability, or trigger a fundamental shift to an alternative state (Scheffer et al., 2001; Folke et al., 2004). Within a diverse functional group, there is the potential for some species to respond positively to environmental change, providing a measure of redundancy and 'insurance' in the event of species loss (Yachi & Loreau, 1999; Loreau et al., 2001). Many studies globally have focused on regions where mussels occur in dense, multi-species beds (Haag, 2012). In such places, the decline of one mussel species can significantly alter ecosystem functioning and associated ecosystem services (Spooner & Vaughn, 2008; Vaughn, 2010). For med-regions, the lack of redundancy in this group means that the extinction of one mussel species would often result in the complete loss of all mussel-derived ecosystem services. Given the importance of freshwater resources to people living in med-regions, this increases the urgency to identify and undertake appropriate conservation and restoration activities for this component of freshwater biodiversity. Research effort to date has been unevenly spread among these regions, and more work is needed in the southern hemisphere in particular (Ball et al., 2013; de Figueroa et al., 2013; de Moor & Day, 2013). The potential for recovery for many species may be limited at present due to the incomplete understanding of the threats faced, and in many cases the original cause of decline is unknown.

The second implication is that, although many rivers in med-regions are not rich in mussel species, there is likely to be considerable intrinsic and extrinsic variability associated with each species (Ferreira-Rodriguez et al., 2019). In SWA for example, *W. carteri* comprises multiple highly divergent, mostly non-

overlapping lineages, and the genetic diversity within those lineages is not evenly spread across the landscape (factors intrinsic to the species) (Benson et al., in review). At the same time, the landscape is not uniform in terms of suitable habitat, threatening processes, and areas of refuge (from short-term disturbances) and refugia (for persistence at evolutionary timescales) (factors extrinsic to the species). At present, *W. carteri* has been assessed as a single conservation unit over the whole of its geographic range. The coarse resolution of this assessment cannot account for intrinsic and extrinsic factors that should be central to the conservation of the species. That being the case, the current conservation advice for this med-mussel is inadequate. This species is listed as Vulnerable based on a 49% reduction in distribution (Threatened Species Scientific Committee, 2017; Klunzinger & Walker, 2020), but whether its decline is lineage or location specific is unknown, and the limited extent of occurrence of the separate genetic units may in itself warrant a higher conservation listing. At the very least, each of the three genetic units requires independent management if the effectiveness of conservation actions for SWA's only mussel species are to be maximised. Further, some locations (e.g. Margaret River, the Blackwood Basin, and the Shannon Basin) should be prioritised for management as they contribute significantly to the intraspecific genetic diversity of *W. carteri*, and may be key areas of climate refugia for freshwater biodiversity in general.

Similar phylogeographic studies are required for other med-regions to describe the distribution of hotspots of intraspecific genetic diversity and location of potential climate refugia. Many mussels found in these regions are similarly wide-ranging as *W. carteri*, and specific populations or areas that are of conservation concern may also require independent management; however, the spatial resolution of data available for conservation assessments is currently lacking for most species. In particular, the level of imperilment for med-mussel species that are wide-ranging beyond their med-boundary is far less than for those confined to med-regions, but many of those are experiencing declines within the med-distribution of their range (e.g. Bogan & Cummings, 2011; Howard et al., 2015; Kristensen et al., 2017). 'Global' conservation assessments may be inadequate for the conservation of such species and med-region specific assessments are required. In the Mediterranean Basin, recent phylogeographic studies have helped to untangle the complex taxonomy of mussels in the region, revealing numerous spatially isolated and divergent lineages that warrant consideration as separate species (Froufe et al., 2016; Araujo et al., 2017; Froufe et al., 2017). These studies are invaluable for the management of med-mussels, as conservation policies and actions are generally applied at the species level (Ferreira-Rodriguez et al., 2019). The recently identified lineages within *W. carteri* may warrant similar species-



level consideration (Klunzinger et al., 2021); further taxonomic investigation incorporating genetic, morphological, and ecological characteristics is required.

The third implication is that mussel populations may actually begin to recover quite rapidly in response to the mitigation of threatening processes at both localised and catchment scales. In SWA, the demographics of mussel populations in the Goodga and Kent rivers suggest that new recruits began to recolonise formerly extirpated reaches almost as soon as they became accessible or once again suitable as habitat (Chapters 3 and 4 respectively). This was particularly noticeable at Goodga River as the installation of the fishway can be precisely dated, and that date matches with the beginning of consistent recruitment throughout the upper reach. The similarity in the level of recent recruitment upstream and downstream of the fishway also suggests that the population may fully recover in the upper reach within the life span of this species. In contrast, mussel population recovery in the Kent River has not been nearly as extensive, being limited to two sites around the Styx River confluence. Although the inferred age of the individuals found at those sites suggests that recruitment coincided with salinity reversal, some cohorts were lacking at those sites, which could reflect annual salinity fluctuations in the river. It also remains to be seen how far upstream recolonisation will occur, as further expansion may be limited by other intrinsic and extrinsic factors, such as increasing aridification and acidification, as well as the salt-tolerance of host-fish and effects on fish-mussel interactions. The impact that these threats have on the connectivity of mussel populations and their hosts remains a key knowledge gap for conservation practitioners. Salinised reaches and increasing intermittence of rivers constitute barriers to dispersal that could lead to increased risk of extinction for some populations. The degree of connectivity within and among med-mussel populations could be assessed using faster evolving genes and microsatellites.

The fourth implication is that the high level of imperilment of med-mussels, in combination with their acknowledged importance as a functional group, makes them especially suitable for a prominent role in the management of freshwater biodiversity. Med-mussels are ideal 'surrogate' species in various forms (e.g. 'flagships', 'indicators', and 'umbrellas'), making them a useful tool to achieve broad conservation outcomes from an ostensibly narrow focus (Caro, 2010; Geist, 2010; Lopes-Lima et al., 2020). Many med-mussels may be useful as umbrella species for conservation, being wide-ranging and having the complex habitat requirements that are a pre-requisite for that role (Benson et al. 2021). Conservation actions directed at such species are expected to benefit numerous co-occurring taxa over a large area (Caro, 2010; Kalinkat et al., 2017). As a flagship, mussels may not be immediately obvious as they lack

the 'charisma' (at least in some people's minds) that is traditionally associated with this role. However, the main role of a flagship is to increase public awareness and raise funds for conservation (Verissimo, MacMillan & Smith, 2011), and for this mussels may actually be well suited. Historically undervalued by environmental decision makers, there is increasing recognition of the various ecological, cultural, and societal benefits mussels provide (Strayer, 2017). Additional ground might be gained if the ecosystem services they provide could be more precisely quantified and better articulated to the human societies that benefit from them (Vaughn, 2018). Surprisingly, few med-mussels have been studied in terms of provision of ecosystem services. A notable exception, filter feeding by *Diplodon chilensis* has been shown to improve water quality in urban lakes (Fuentealba & Henriquez, 2009), fish farms (Parada et al., 2008) and rural wells (Lara, Contreras & Encina, 2002). More studies in this vein could highlight the important role that mussels play within a med-region context.

Med-mussels may also be a useful 'indicator' species for the recovery of biodiversity. Mussels are well suited for use as indicator species in environmental assessments due to their complex lifecycle and sensitivity to disturbance (Geist, 2010); however, for most species basic ecological knowledge that is a pre-requisite for this role is lacking (Carignan & Villard, 2002; Lopes-Lima et al., 2020). While they have been recommended as indicators of environmental decline before (Geist, 2010; Lopes-Lima et al., 2020), and are often utilised as bio-indicators to monitor the quality of aquatic habitat (Pander & Geist, 2013), there do not appear to be any instances of mussels being recommended as indicators of biodiversity recovery specifically. Obvious reasons for this include their slow growth rate, cryptic nature, and the difficulty in detecting juvenile mussels (Hastie et al., 2000). While these factors will limit their usefulness for this role in many cases, mussels may be helpful in indicating longer-term recovery of biodiversity (e.g. Sietman et al., 2001; Riccardi et al., 2016; Gillis et al., 2017). Further, their external annuli can describe post recovery demographics, and allow inferences to be made about the time of first recolonisation and fluctuations in recruitment during the recovery process (Benson et al., 2018; Benson et al., 2019). Research into all aspects of med-mussel biology and ecology will increase their utility as indicator species (Carignan & Villard, 2002). Therefore, research that addresses knowledge gaps for a med-mussel species serves the dual purpose of (1) aiding the conservation of an imperiled taxon, and (2) potentially providing a useful tool for the conservation of freshwater biodiversity in general.

In closing, it is worth noting that mussels were not the target of restoration actions undertaken in SWA, and *W. carteri* has instead 'hitch-hiked' the road to recovery in the Goodga and Kent rivers.

Nonetheless, the observed recovery in these locations provides examples of freshwater biodiversity

benefiting from improved management practices in aquatic systems and the terrestrial landscape. Importantly, this provides rare success stories that may help to bring mussels into the public consciousness and secure funding for future conservation and restoration efforts. This would not only benefit med-mussel species, but also freshwater biodiversity in general due to the numerous ecosystem engineering roles they play (Vaughn & Hakenkamp, 2001; Geist, 2010; Geist & Hawkins, 2016). This thesis has demonstrated that there is an urgent need to increase conservation effort aimed at mussels in med-regions. Med-mussel populations in 'pristine' systems hold great value, as those areas are likely to have important environmental resources that buffer mussel populations against future disturbances. However, as the majority of med-regions are highly altered, most conservation opportunities will involve mitigating threats in degraded habitats. The fact that mussel populations can *begin* to recover quite rapidly increases the incentive to commence restoration actions on declining populations; however, the time to 'full' recovery is unknown and this has yet to be observed. This highlights that delaying the implementation of restoration actions will only prolong what is likely to be a long recovery process, and that prevention is better than cure in the case of mussel conservation

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Appendix 1:  
Supplementary material for chapter 2



***Cover picture: Fifty-year storm at Goodga River***

**Appendix 1:** Freshwater mussels known to occur within med-region boundaries. Conservation information is based on the IUCN Red List, while distribution is based on the Red List and additional references. Where relevant, columns 2-4 show details of separate Red List assessments for each species. Scope of assessment refers to Global (Glo), Europe (Euro), Med (Med), Northern Africa (NA), Southern Africa (SA), and Pan Africa (PA). In column 5 species from the Mediterranean basin (MB) are also divided into subregions of Europe (Eur), Northern Africa (NA), and Middle-east (ME)

| Species                        | Scope of Assessment | Red List Status (year) | Population Trend         | Region (subregion) | Distribution within med-region   | References (Red List citations in bold)  |
|--------------------------------|---------------------|------------------------|--------------------------|--------------------|--|--|
| <i>Anodonta anatina</i>        | Glo<br>Eur          | LC (2013)<br>LC (2010) | Decreasing<br>Unknown    | MB (Euro/ME/NA)    | Widespread in MB-Euro and Morocco, with a more restricted distribution in the rest of the MB | <b>Lopes-Lima (2014c)</b><br><b>Killeen &amp; Vavrova (2011)</b><br>Gomes-dos-Santos et al. (2019)<br>Lopes-Lima et al. (2017b)<br>Smith et al. (2014) |
| <i>Anodonta cygnea</i>         | Glo<br>Eur          | LC (2013)<br>NT (2010) | Decreasing<br>Decreasing | MB (Euro/ME)       | Widespread though patchy. Possibly present in MB-NA (requires genetic confirmation)          | <b>Lopes-Lima (2014a)</b><br><b>Killeen &amp; Aldridge (2011)</b><br>Lopes-Lima et al. (2017b)<br>Smith et al. (2014)                                  |
| † <i>Anodonta exulcerata</i>   | -                   | -                      | -                        | MB (Euro)          | Widespread in Italy and parts of coastal Croatia.  | Froufe et al. (2017)   |
| † <i>Anodonta pseudodopsis</i> | Glo                 | EN (2013)              | Unknown                  | MB (ME)            | Restricted to the Orontes Basin in Syria, Turkey and possibly Lebanon                        | <b>Lopes-Lima &amp; Seddon (2014a)</b><br>Smith et al. (2014)  |
| <i>Anodonta vescoiana</i>      | Glo                 | NT (2013)              | Unknown                  | MB (ME)            | Only known from Lake Gölbaşı on the lower Orontes in Turkey                                  | <b>Lopes-Lima (2014b)</b><br>Smith et al. (2014)   |

| Species                            | Scope of Assessment | Red List Status (year) | Population Trend         | Region (subregion) | Distribution within med-region   | Red List citations (in bold) and additional references   |
|------------------------------------|---------------------|------------------------|--------------------------|--------------------|--|--|
| † <i>Leguminaia saulcyi</i>        | Glo                 | CR (2013)              | Unknown                  | MB (ME)            | Syria, Turkey, and Jordan. Records outside of the MB-ME likely erroneous. Believed extinct in Israel.                  | <b>Lopes-Lima &amp; Seddon (2014b)</b><br>Smith et al. (2014)  |
| <i>Leguminaia wheatleyi</i>        | Glo                 | NT (2013)              | Decreasing               | MB (ME)            | Extant in upper Euphrates and Tigris catchment in Syria and Turkey. Believed extinct in Israel and possibly Palestine. | <b>Lopes-Lima &amp; Kebapçı (2014a)</b><br>Smith et al. (2014)   |
| <i>Pseudunio auricularius</i>      | Glo, Eur & Med      | CR (2010)              | Decreasing               | MB (Euro)          | Formerly widespread in Europe, now restricted to 4 watersheds in non-med France, and 1 river in Spain                  | <b>Prie (2010)</b><br>Prié et al. (2018)<br>Lopes-Lima et al. (2017b)                                  |
| † <i>Pseudunio homsensis</i>       | Glo & Med           | EN (2013)              | Unknown                  | MB (ME)            | Restricted to the Orontes Basin and possibly rivers nearby. Currently known from 1 living individual                   | <b>Lopes-Lima &amp; Kebapçı (2014b)</b><br>Vikhrev et al. (2018)                                       |
| <i>Margaritifera margaritifera</i> | Glo<br>Eur          | EN (2010)<br>CR (2010) | Decreasing<br>Decreasing | MB (Euro)          | A Holarctic species that is declining in Europe including in MB-Euro   | <b>Moorkens et al. (2011)</b><br><b>Moorkens (2011)</b><br>Lopes-Lima et al. (2017b)                   |
| † <i>Pseudunio maroccanus</i>      | Glo, NA & PA        | CR (2007)              | Decreasing               | MB (NA)            | Endemic to MB-NA, restricted to just a few locations in Morocco following dramatic recent decline                      | <b>Van Damme &amp; Ghamizi (2010a)</b><br>Gomes-dos-Santos et al. (2019)<br>Sousa et al. (2016, 2018b) |
| † <i>Microcondylaea bonellii</i>   | Glo, Eur & Med      | VU (2009)              | Decreasing               | MB (Euro)          | Endemic to the Italian Peninsula and coastal rivers of the Balkans   | <b>Albrecht et al. (2011)</b><br>Froufe et al. (2017)<br>Lopes-Lima et al. (2017b)                     |

| Species                                   | Scope of Assessment | Red List Status (year)              | Population Trend                    | Region (subregion) | Distribution within med-region   | Red List citations (in bold) and additional references  |
|---|---------------------|-------------------------------------|-------------------------------------|--------------------|--|---|
| <i>Potomida littoralis</i> <sup>1</sup>   | Glo<br>Eur          | EN (2013)<br>NT (2010)              | Decreasing<br>Decreasing            | MB (Euro/NA)       | Widespread in the Iberian Peninsula in MB-Euro, as well as in MB-NA                                    | <b>Lopes-Lima, Prie &amp; Seddon al. (2014)</b><br><b>Seddon (2011)</b><br>Gomes-dos-Santos et al. (2019)<br>Froufe et al. (2016b)<br>Araujo et al. (2016)                              |
| † <i>Potomida acarnanica</i> <sup>1</sup> | -                   | -                                   | -                                   | MB (Euro)          | Restricted to Greece   | Froufe et al. (2016b)<br>Araujo et al. (2016)   |
| † <i>Potomida semirugata</i> <sup>1</sup> | -                   | -                                   | -                                   | MB (ME)            | Various countries of the MB-ME. Potentially a subspecies of <i>P. acarnanica</i> (Araujo et al., 2016) | Froufe et al. (2016b)   |
| <i>Unio crassus</i> <sup>2</sup>          | Glo<br>Eur<br>Med   | EN (2013)<br>VU (2009)<br>DD (2009) | Decreasing<br>Decreasing<br>Unknown | MB (Euro/ME)       | Historically very wide ranging. The distribution in the MB is more limited (mainly Greece and Turkey)  | <b>Aldridge, Fehér &amp; von Proschwitz (2011)</b><br><b>Lopes-Lima, Kebapçı &amp; Van Damme (2014a)</b><br><b>Vavrova (2011a)</b><br>Lopes-Lima et al. (2017b)<br>Araujo et al. (2017) |
| † <i>Unio bruguierianus</i> <sup>2</sup>  | -                   | -                                   | -                                   | MB (Euro/ME)       | Restricted to the Axios and Pinios rivers in eastern Greece  | Lopes-Lima et al. (2017b)<br>Araujo et al. (2017)   |
| † <i>Unio ionicus</i> <sup>2</sup>        | -                   | -                                   | -                                   | MB (Euro)          | Albania and Western Greece   | Araujo et al. (2017)  |

| Species                                  | Scope of Assessment | Red List Status (year) | Population Trend         | Region (subregion) | Distribution within med-region   | Red List citations (in bold) and additional references  |
|--|---------------------|------------------------|--------------------------|--------------------|--|---|
| <i>Unio mancus</i>                       | Glo<br>Eur          | NT (2013)<br>NT (2009) | Decreasing<br>Decreasing | MB (Euro/ME)       | France, Italy, and Spain, based on revisions by Araujo et al. (2017). Also, Turkey, Lebanon, and Syria based on Red list and Smith et al. (2014) | <b>Lopes-Lima &amp; Seddon (2014e)</b><br><b>Araujo (2011c)</b><br>Araujo et al. (2017)<br>Smith et al. (2014)            |
| † <i>Unio elongatulus</i>                | -                   | -                      | -                        | MB (Euro)          | Northern Italy and Croatia   | Lopes-Lima et al. (2017b)<br>Araujo et al. (2017)<br>Froufe et al. (2017)   |
| † <i>Unio delphinus</i> <sup>3</sup>     | Glo & Eur           | NT (2009)              | Decreasing               | MB (Euro)          | Widespread in basins in the western half of the Iberian Peninsula  | <b>Araujo (2011a)</b><br>Lopes-Lima et al. (2017b)<br>Araujo et al. (2017)<br>Froufe et al. (2016a)                       |
| † <i>Unio foucauldianus</i> <sup>3</sup> | Glo, NA & PA        | CR (2007)              | Decreasing               | MB (NA)            | Patchy distribution in 12 Moroccan river basins, most draining to the Atlantic. More widely distributed than previously thought                  | <b>Van Damme &amp; Ghamizi (2010c)</b><br>Gomes-dos-Santos et al. (2019)<br>Araujo et al. (2017)<br>Froufe et al. (2016a) |
| † <i>Unio durieui</i>                    | Glo, NA & PA        | EN (2007)              | Decreasing               | MB (NA)            | Morocco, Algeria and Tunisia   | <b>Van Damme &amp; Ghamizi (2010b)</b><br>Araujo et al. (2017)  |
| † <i>Unio ravoisieri</i>                 | -                   | -                      | -                        | MB (Euro/NA)       | Algeria and Tunisia with disjunct populations in northeast Spain   | Araujo et al. (2017)  |
| † <i>Unio gibbus</i>                     | Eur                 | CR (2009)              | Unknown                  | MB (Euro/NA)       | Highly fragmented distribution from Morocco to Tunisia. One small population in southern Spain   | <b>Araujo (2011b)</b><br>Gomes-dos-Santos et al. (2019)<br>Araujo et al. (2017)<br>Froufe et al. (2016a)                  |

| Species                               | Scope of Assessment | Red List Status (year) | Population Trend | Region (subregion) | Distribution within med-region   | Red List citations (in bold) and additional references                                |
|---------------------------------------|---------------------|------------------------|------------------|--------------------|--|---|
| <i>Unio pictorum</i>                  | Glo                 | LC (2011)              | Unknown          | MB (Euro)          | The most widely distributed member of its genus. Generally not found in MB, but its range extends to Greece and Turkey   | <b>Van Damme (2011a)</b>  |
|                                       | Eur                 | LC (2010)              | Unknown          |                    |  | <b>Vavrova (2011b)</b><br>Araujo et al. (2017)<br>Froufe et al. (2016a)               |
| <i>Unio tigridis</i> <sup>4</sup>     | Glo & Med           | LC (2013)              | Decreasing       | MB (ME)            | Turkey and Syria on the lower Orontes and the Euphrates-Tigris basin   | <b>Lopes-Lima &amp; Seddon (2014d)</b><br>Araujo et al. (2017)<br>Smith et al. (2014) |
| † <i>Unio terminalis</i> <sup>4</sup> | Glo & Med           | VU (2013)              | Decreasing       | MB (ME)            | Syria, Lebanon and Israel (Orontes and Jordan River Basins; likely lost from coastal rivers). Maintained under <i>U. tigridis</i> by Araujo et al. (2017) pending further analysis | <b>Lopes-Lima &amp; Seddon (2014c)</b><br>Smith et al. (2014)                         |
| † <i>Unio tumidiformis</i>            | Glo, Eur & Med      | VU (2009)              | Decreasing       | MB (Euro)          | Spain and Portugal on the Southwestern Iberian Peninsula.  | <b>Araujo (2011d)</b><br>Araujo et al. (2017)   |
| <i>Unio tumidus</i>                   | Glo                 | LC (2011)              | Unknown          | MB (Euro)          | Very widely distributed species but generally absent from MB. Listed as extant in countries including Spain, Albania, Greece on the Red List                                       | <b>Van Damme (2011b)</b>  |
|                                       | Eur                 | LC (2010)              | Decreasing       |                    |  | <b>Vavrova (2011c)</b><br>Araujo et al. (2017)<br>Lopes-Lima et al. (2017b)           |



| Species   | Scope of Assessment | Red List Status (year) | Population Trend | Region (subregion) | Distribution within med-region   | Red List citations (in bold) and additional references  |
|---|---------------------|------------------------|------------------|--------------------|--|---|
| <i>Anodonta nuttalliana</i> <sup>5</sup>            | Glo                 | VU (2016)              | Decreasing       | CCali              | Historical distribution and abundance records are confounded by morphological similarity to other Anodontines. Not detected in any historical sites in California in 2008-09 | <b>Blevins et al. (2016a)</b><br>Blevins et al. (2017)<br>Howard et al. (2015)<br>Mock et al. (2010)                            |
| <i>Anodonta californiensis</i> <sup>5</sup>         | -                   | -                      | -                | CCali              | As above   | As above  |
| <i>Gonidea angulata</i>                             | Glo                 | VU (2016)              | Decreasing       | CCali              | Known historical distribution is patchy in CCali. Not detected in any historical sites in southern California in 2008-09   | <b>Blevins et al. (2016b)</b><br>Blevins et al. (2017)<br>Howard et al. (2015)  |
| <i>Margaritifera falcata</i>                        | Glo                 | NT (2016)              | Decreasing       | CCali              | Known historical distribution is patchy in CCali. Not detected in >40% of historical sites in California in 2008-09.   | <b>Blevins et al. (2016c)</b><br>Blevins et al. (2017)<br>Howard et al. (2015)  |
| <i>Diplodon chilensis</i>                           | Glo                 | LC (2011)              | Unknown          | CChile             | Widespread and often abundant, with recent declines including extirpations   | <b>Bogan &amp; Cummings (2011)</b><br>Pereira et al. (2014)<br>Fuentealba, Figueroa & Morrone (2010)                            |
| <i>Unio caffer</i><br>(syn <i>Cafferia caffer</i> ) | Glo, PA & SA        | LC (2007)              | Unknown          | SAfr               | Historical distribution rarely reported. Museum records indicate presence in CFR rivers, where populations have declined dramatically including extirpations                 | <b>Kristensen, Stensgaard, &amp; Appleton (2017)</b><br>Sonamzi et al. (2019)<br>Graf & Cummings (2011)<br>Day & de Moor (2002) |

| Species                         | Scope of Assessment | Red List Status (year) | Population Trend | Region (subregion) | Distribution within med-region   | Red List citations (in bold) and additional references  |
|---------------------------------|---------------------|------------------------|------------------|--------------------|--|---|
| † <i>Westralunio carteri</i>    | Glo                 | VU (2014)              | Decreasing       | SWA                | Widespread within 50-100 km of the coast, with a 49% decline in EOO in the past 3 generations.                                     | <b>Klunzinger &amp; Walker (2014)</b><br>Klunzinger et al. (2015)<br>Walker, Jones & Klunzinger (2014a)                                 |
| <i>Alathyria jacksoni</i>       | Glo                 | DD (2011)              | Unknown          | SA                 | Endemic to the Murray River where it is patchily distributed. Populations have declined in the lower reach which passes through SA | <b>Köhler (2011)</b><br>Walker, Jones & Klunzinger (2014a)<br>Jones & Byrne (2014)<br>Playford & Walker (2008)                          |
| † <i>Hyridella glenelgensis</i> | Glo                 | CR (2014)              | Decreasing       | SA                 | Restricted to one tributary of the Glenelg River in Victoria, Australia.   | <b>Walker, Jones &amp; Klunzinger (2014b)</b><br>Walker, Jones & Klunzinger (2014a)<br>Jones & Byrne (2014)<br>Playford & Walker (2008) |
| <i>Hyridella narracanensis</i>  | Glo                 | NT (2014)              | Decreasing       | SA                 | Occurs mostly outside SA, restricted to one location in the med-region   | <b>Klunzinger, Walker &amp; Jones (2014)</b><br>Walker, Jones & Klunzinger (2014a)<br>Jones & Byrne (2014)<br>Playford & Walker (2008)  |
| <i>Velesunio ambiguus</i>       | -                   | -                      | -                | SA                 | Widespread, but more common in inland waters. Patchily distributed in coastal rivers such as those in SA                           | Walker, Jones & Klunzinger (2014a)<br>Jones & Byrne (2014)<br>Playford & Walker (2008)  |

†Species is endemic to med-region

Species sharing a superscript number were recently split taxonomically or are often considered synonymous

Appendix 2:  
Supplementary information for chapter 5



*Cover picture: Winter flood at Millar's basin on the Kent River*

**Table S1:** Primers, annealing temperature and MgCl<sub>2</sub> concentration for the mtDNA and nuclear genes collected during this study. Nuclear genes were not included in the analysis

| Gene | Primer      | Sequence (5'-3')                   | Reference                      | Length (bp) | Annealing Temp (°C) | MgCl <sub>2</sub> Conc |
|------|-------------|------------------------------------|--------------------------------|-------------|---------------------|------------------------|
| COI  | LCOI        | GGT CAA CAA ATC ATA AAG ATA TTG G  | Folmer et al. (1994)           | 687         | 48                  | 3mM                    |
|      | HCO2        | TAA ACT TCA GGG TGA CCA AAA AAT CA |                                |             |                     |                        |
| 16S  | 16Sar_L_myt | CGA CTG TTT AAC AAA AAC AT         | Lydeard, Mulvey & Davis (1996) | 516-518     | 50                  | 4mM                    |
|      | 16Sbr_L_myt | CCG TTC TGA ACT CAG CTC ATG T      |                                |             |                     |                        |
| 18S  | 1F          | TAC CTG GTT GAT CCT GCC AGT AG     | Giribet et al. (1996)          | 935         | 50                  | 4mM                    |
|      | 5R          | CTT GGC AAA TGC TTT CGC            |                                |             |                     |                        |
| 28S  | 28S_rD1.2a  | CCC SSG TAA TTT AAG CAT ATT A      | Whiting (2002)                 | 804         | 55                  | 2.5mM                  |
|      | D4RB        | TGT TAG ACT CCT TGG TCC GTG T      | Park & Foighil (2000)          |             |                     |                        |

## References

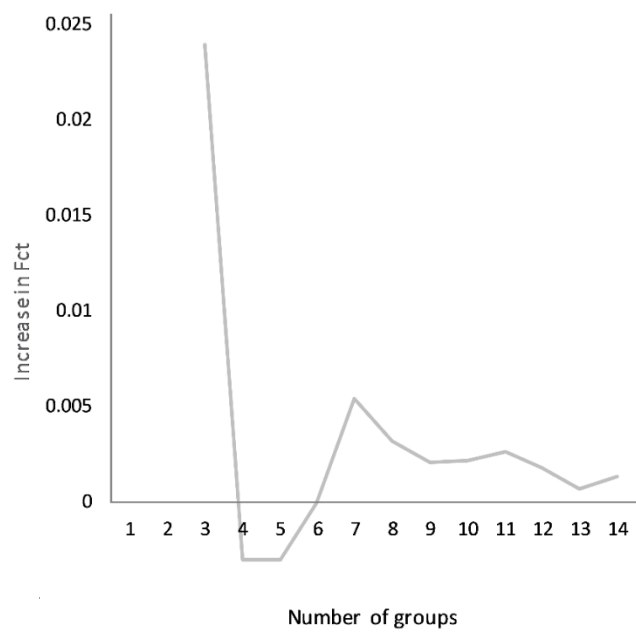
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**Table S2:** COI haplotype distributions for *Westralunio carteri* in southwestern Australia. Basins are arranged from north-south between Moore-Hill and the Capes, and from west-east between Blackwood and Albany Coast. Location refers to the waterbody sampled. Basin abbreviations and location numbers correspond to Figure 2. Haplotypes (numbered in columns) are arranged by haplogroup, and correspond to the networks in Figure 4. Sample size (n) and haplotype diversity (h) are also shown. Note the WcIII haplogroup is placed between WcI and WcII to reflect its intermediate location

| Basin               | Location         | n   | Haplotypes     |   |   |   |   |    |   |   |    |    |    |    |    |                  |    |    |    |    |    |    |    |    |    |                 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | h |   |   |   |   |   |
|---------------------|------------------|-----|----------------|---|---|---|---|----|---|---|----|----|----|----|----|------------------|----|----|----|----|----|----|----|----|----|-----------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|---|---|---|---|---|---|
|                     |                  |     | WcI haplogroup |   |   |   |   |    |   |   |    |    |    |    |    | WcIII haplogroup |    |    |    |    |    |    |    |    |    | WcII haplogroup |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |   |   |   |   |   |
|                     |                  |     | 1              | 2 | 3 | 4 | 5 | 6  | 7 | 8 | 9  | 10 | 11 | 12 | 13 | 14               | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24              | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 | 43 | 44 | 45 | 46 |   |   |   |   |   |   |
| Moore-Hill (MH)     | Gingin (1)       | 1   | 1              |   |   |   |   |    |   |   |    |    |    |    |    |                  |    |    |    |    |    |    |    |    |    |                 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |   | 1 |   |   |   |
| Swan (Sw)           | Marbling (2)     | 1   |                | 1 |   |   |   |    |   |   |    |    |    |    |    |                  |    |    |    |    |    |    |    |    |    |                 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |   |   |   | 1 |   |
|                     | Neerigen (3)     | 1   |                |   | 1 |   |   |    |   |   |    |    |    |    |    |                  |    |    |    |    |    |    |    |    |    |                 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |   |   |   | 1 |   |
|                     | Wungong (4)      | 3   |                |   |   | 2 | 1 |    |   |   |    |    |    |    |    |                  |    |    |    |    |    |    |    |    |    |                 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |   |   |   | 2 |   |
|                     | Lesch (5)        | 1   |                |   | 1 |   |   |    |   |   |    |    |    |    |    |                  |    |    |    |    |    |    |    |    |    |                 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |   |   |   | 1 |   |
| Murray (Mu)         | Serpentine (6)   | 6   |                |   |   |   |   | 1  | 4 | 1 |    |    |    |    |    |                  |    |    |    |    |    |    |    |    |    |                 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |   |   | 3 |   |   |
| Collie-Preston (CP) | Collie (7)       | 6   |                |   |   |   |   | 3  |   |   | 1  | 1  | 1  |    |    |                  |    |    |    |    |    |    |    |    |    |                 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |   |   | 4 |   |   |
|                     | Preston (8)      | 5   |                |   |   |   |   | 4  |   |   |    |    |    |    | 1  |                  |    |    |    |    |    |    |    |    |    |                 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |   |   | 2 |   |   |
| Busseton North (BN) | Capel (9)        | 5   |                |   |   |   |   | 5  |   |   |    |    |    |    |    |                  |    |    |    |    |    |    |    |    |    |                 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |   |   |   | 1 |   |
|                     | Abba (10)        | 5   |                |   |   |   |   | 4  |   |   | 1  |    |    |    |    |                  |    |    |    |    |    |    |    |    |    |                 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |   |   |   | 2 |   |
|                     | Ludlow (11)      | 5   |                |   |   |   |   | 4  |   |   |    |    |    |    | 1  |                  |    |    |    |    |    |    |    |    |    |                 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |   |   |   | 2 |   |
|                     | Carbanup (12)    | 5   | 1              |   |   |   |   | 4  |   |   |    |    |    |    |    |                  |    |    |    |    |    |    |    |    |    |                 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |   |   |   | 2 |   |
| Capes               | Wilyabrup (13)   | 5   |                |   |   |   |   |    |   |   | 5  |    |    |    |    |                  |    |    |    |    |    |    |    |    |    |                 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |   |   |   | 1 |   |
|                     | Ellensbrook (14) | 5   |                |   |   |   |   |    |   |   | 5  |    |    |    |    |                  |    |    |    |    |    |    |    |    |    |                 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |   |   |   |   | 1 |
|                     | Margaret (15)    | 10  |                |   |   |   |   | 1  |   |   |    |    |    |    | 1  | 4                | 1  | 1  | 1  | 1  |    |    |    |    |    |                 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |   |   |   | 7 |   |
|                     | Boodjiup (16)    | 5   |                |   |   |   |   |    |   |   | 4  |    |    |    |    |                  |    |    |    |    |    |    |    |    | 1  |                 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |   |   |   | 2 |   |
| Blackwood (Bw)      | Scott (17)       | 5   |                |   |   |   |   |    |   |   |    |    |    |    |    |                  |    |    |    |    | 1  |    |    |    |    |                 |    | 2  | 1  | 1  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |   |   | 4 |   |   |
|                     | Chapman (18)     | 5   |                |   |   |   |   |    |   |   |    |    |    |    |    |                  |    |    |    |    |    | 1  | 1  |    |    |                 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |   |   |   |   | 4 |
|                     | St Johns (19)    | 8   |                |   |   |   |   |    |   |   |    |    |    |    |    |                  |    |    |    |    |    |    |    | 1  |    | 4               |    |    | 1  | 2  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |   |   |   | 4 |   |
| Donnelly (Do)       | Donnelly (20)    | 5   |                |   |   |   |   |    |   |   |    |    |    |    |    |                  |    |    |    |    |    |    |    |    | 5  |                 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |   |   | 1 |   |   |
| Warren (Wa)         | Yeagerup (21)    | 5   |                |   |   |   |   |    |   |   |    |    |    |    |    |                  |    |    |    |    |    |    |    |    |    |                 |    |    |    |    |    |    | 1  | 1  | 2  | 1  |    |    |    |    |    |    |    |    |    |    |    |    |   |   |   |   | 4 |   |
|                     | Warren (22)      | 5   |                |   |   |   |   |    |   |   |    |    |    |    |    |                  |    |    |    |    |    |    |    |    |    |                 |    |    |    |    |    |    |    |    |    | 3  | 1  | 1  |    |    |    |    |    |    |    |    |    |    |   |   |   |   | 3 |   |
| Shannon (Sh)        | Gardner (23)     | 5   |                |   |   |   |   |    |   |   |    |    |    |    |    |                  |    |    |    |    |    |    |    |    |    |                 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | 4  | 1  |    |    |   |   |   | 2 |   |   |
|                     | Shannon (24)     | 5   |                |   |   |   |   |    |   |   |    |    |    |    |    |                  |    |    |    |    |    |    |    |    |    |                 |    |    |    |    |    |    |    |    |    | 1  |    | 1  |    |    |    |    |    | 1  | 2  |    |    |    |   |   | 4 |   |   |   |
|                     | Inlet (25)       | 4   |                |   |   |   |   |    |   |   |    |    |    |    |    |                  |    |    |    |    |    |    |    |    |    |                 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |   |   | 2 |   |   |
| Frankland-Deep (FD) | Deep (26)        | 5   |                |   |   |   |   |    |   |   |    |    |    |    |    |                  |    |    |    |    |    |    |    |    |    |                 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |   | 3 |   |   |   |
|                     | Walpole (27)     | 5   |                |   |   |   |   |    |   |   |    |    |    |    |    |                  |    |    |    |    |    |    |    |    |    |                 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |   |   |   | 1 |   |
| Kent River (Ke)     | Bow (28)         | 5   |                |   |   |   |   |    |   |   |    |    |    |    |    |                  |    |    |    |    |    |    |    |    |    |                 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |   |   | 5 |   |   |
|                     | Kent (29)        | 12  |                |   |   |   |   |    |   |   |    |    |    |    |    |                  |    |    |    |    |    |    |    |    |    |                 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |   |   | 1 | 4 |   |
| Denmark (De)        | Marbellup (30)   | 5   |                |   |   |   |   |    |   |   |    |    |    |    |    |                  |    |    |    |    |    |    |    |    |    |                 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |   |   | 1 |   |   |
| Albany (Al)         | Goodga (31)      | 9   |                |   |   |   |   |    |   |   |    |    |    |    |    |                  |    |    |    |    |    |    |    |    |    |                 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |   |   | 2 |   |   |
|                     | Waychinicup (32) | 7   |                |   |   |   |   |    |   |   |    |    |    |    |    |                  |    |    |    |    |    |    |    |    |    |                 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |   |   |   | 3 |   |
|                     | Total            | 164 | 2              | 1 | 2 | 2 | 1 | 26 | 4 | 1 | 16 | 1  | 1  | 1  | 1  | 1                | 4  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 6               | 6  | 1  | 1  | 3  | 2  | 2  | 1  | 1  | 6  | 2  | 8  | 20 | 1  | 19 | 2  | 3  | 1  | 2  | 1  | 1  | 1  | 1  | 1 | 1 |   |   |   |   |

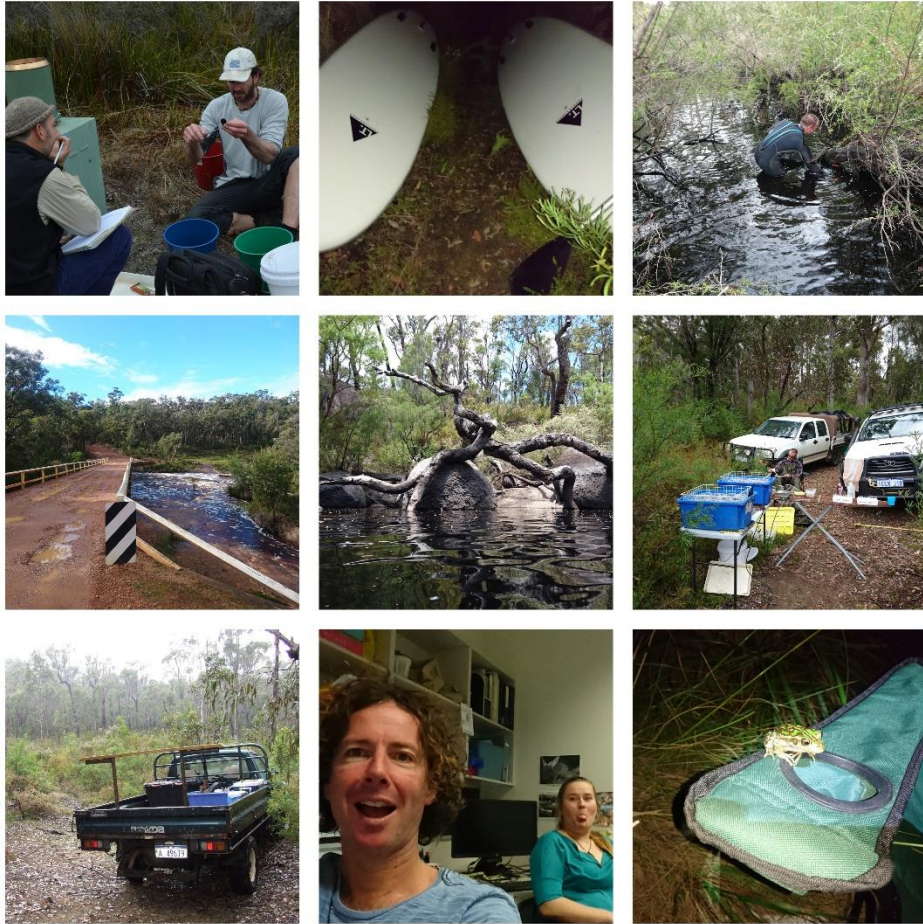
**Table S3:** 16S haplotype distributions for *Westralunio carteri* in southwestern Australia. Basins are arranged from north-south between Swan Coast and the Capes, and from west-east between Blackwood and Albany Coast. Location refers to the waterbody sampled. Basin abbreviations and location numbers correspond to Figure 2. Haplotypes (numbered in columns) are arranged by haplogroup, and correspond to the networks in Figure 4. Sample size (n) and haplotype diversity (h) are also shown

| Basin                | Location         | n   | Haplotype      |   |   |   |                    |   |    |    |   |    |    |    |    |    |    |    | h |
|----------------------|------------------|-----|----------------|---|---|---|--------------------|---|----|----|---|----|----|----|----|----|----|----|---|
|                      |                  |     | Wcl haplogroup |   |   |   | Wcl-III haplogroup |   |    |    |   |    |    |    |    |    |    |    |   |
|                      |                  |     | 1              | 2 | 3 | 4 | 5                  | 6 | 7  | 8  | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |   |
| Swan (Sw)            | Neerigen (3)     | 1   | 1              |   |   |   |                    |   |    |    |   |    |    |    |    |    |    |    | 1 |
| "                    | Lesch (5)        | 1   |                | 1 |   |   |                    |   |    |    |   |    |    |    |    |    |    |    | 1 |
| Murray (Mu)          | Serpentine (6)   | 1   | 1              |   |   |   |                    |   |    |    |   |    |    |    |    |    |    |    | 1 |
| Collie-Preston (CP)  | Collie (7)       | 1   | 1              |   |   |   |                    |   |    |    |   |    |    |    |    |    |    |    | 1 |
| Busselton North (BN) | Capel (9)        | 5   | 4              |   | 1 |   |                    |   |    |    |   |    |    |    |    |    |    |    | 2 |
| "                    | Abba (10)        | 5   | 5              |   |   |   |                    |   |    |    |   |    |    |    |    |    |    |    | 1 |
| "                    | Ludlow (11)      | 5   | 4              |   |   | 1 |                    |   |    |    |   |    |    |    |    |    |    |    | 2 |
| "                    | Carbanup (12)    | 5   | 5              |   |   |   |                    |   |    |    |   |    |    |    |    |    |    |    | 1 |
| Capes                | Wilyabrup (13)   | 5   | 5              |   |   |   |                    |   |    |    |   |    |    |    |    |    |    |    | 1 |
| "                    | Ellensbrook (14) | 5   | 5              |   |   |   |                    |   |    |    |   |    |    |    |    |    |    |    | 1 |
| "                    | Margaret (15)    | 7   | 2              |   |   |   | 4                  | 1 |    |    |   |    |    |    |    |    |    |    | 3 |
| "                    | Boodjiup (16)    | 5   | 4              |   |   |   |                    |   | 1  |    |   |    |    |    |    |    |    |    | 2 |
| Blackwood (Bw)       | Scott (17)       | 5   |                |   |   |   | 1                  |   | 4  |    |   |    |    |    |    |    |    |    | 2 |
| "                    | Chapman (18)     | 5   |                |   |   |   | 1                  |   | 1  | 1  | 2 |    |    |    |    |    |    |    | 4 |
| "                    | St Johns (19)    | 6   |                |   |   |   |                    |   | 4  |    |   | 1  | 1  |    |    |    |    |    | 3 |
| Donnelly (Do)        | Donnelly (20)    | 5   |                |   |   |   |                    |   | 5  |    |   |    |    |    |    |    |    |    | 1 |
| Warren (Wa)          | Yeagerup (21)    | 5   |                |   |   |   |                    |   | 4  |    |   |    |    | 1  |    |    |    |    | 2 |
| "                    | Warren (22)      | 5   |                |   |   |   |                    |   | 5  |    |   |    |    |    |    |    |    |    | 1 |
| Shannon (Sh)         | Gardner (23)     | 5   |                |   |   |   |                    |   |    | 4  |   |    |    |    | 1  |    |    |    | 2 |
| "                    | Shannon (24)     | 5   |                |   |   |   |                    |   | 3  |    |   |    |    |    |    | 2  |    |    | 2 |
| "                    | Inlet (25)       | 4   |                |   |   |   |                    |   | 3  |    |   |    |    |    |    |    | 1  |    | 2 |
| Frankland-Deep (FD)  | Deep (26)        | 5   |                |   |   |   |                    |   | 1  | 4  |   |    |    |    |    |    |    |    | 2 |
| "                    | Walpole (27)     | 5   |                |   |   |   |                    |   |    | 5  |   |    |    |    |    |    |    |    | 1 |
| Kent (Ke)            | Bow (28)         | 5   |                |   |   |   |                    |   |    | 5  |   |    |    |    |    |    |    |    | 1 |
| "                    | Kent (29)        | 7   |                |   |   |   |                    |   |    | 7  |   |    |    |    |    |    |    |    | 1 |
| Denmark (De)         | Marbellup (30)   | 5   |                |   |   |   |                    |   |    | 5  |   |    |    |    |    |    |    |    | 1 |
| Albany (Al)          | Goodga (31)      | 5   |                |   |   |   |                    |   |    | 5  |   |    |    |    |    |    |    |    | 1 |
| "                    | Waychinicup (32) | 7   |                |   |   |   |                    |   | 6  |    |   |    |    |    |    |    |    | 1  | 2 |
|                      | Total            | 130 | 37             | 1 | 1 | 1 | 6                  | 1 | 37 | 36 | 2 | 1  | 1  | 1  | 1  | 2  | 1  | 1  |   |



**Figure S1:** Spatial Analysis of Molecular Variance. Increase in  $F_{CT}$  in response to number of groups ( $K$ ) (i.e.  $F_{CT}(K) - F_{CT}(K-1)$ )





**Picture:** Various study sites and research assistants